Case Docket No. 107-145

THE HON. COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D. C. 20231

Sir:
Transmitted herewith for filing is the patent application of:

Inventor: A. SAID EL SHAMI

For: METHOD FOR MEASURING FREE LIGANDS IN BIOLOGICAL FLUIDS, AND ASSAY KITS FOR MEASURING SAME

Enclosed are:

- [X] 10 sheet(s) of drawing(s). (Formal)
- [X] An assignment of the invention to: DIAGNOSTIC PRODUCTS CORPORATION
- [X] Certificate of Mailing by Express Mail No. B34445114
- [ ] A certified copy of a _____ application.
- [ ] An associate Power of Attorney.
- [ ] A verified statement to establish small entity status under 37 CFR 1.9 and 37 CFR 1.27.
- [X] Declaration and Power of Attorney

The filing fee has been calculated as shown below:

For:	(Col. 1) No. Filed	(Col. 2) No. Extra
Basic Fee:	[redacted]	
Total Claims	27 -20=	* 7
Indep Claims	2 - 3=	* -0-
[ ] Multiple Dependent Claim Presented		

*If the difference in Col. 1 is less than zero, enter "0" in Col. 2

SMALL ENTITY Rate	Fee		OTHER THAN A SMALL ENTITY Rate	Fee
[redacted]	$150	or	[redacted]	$300
x5=	$	or	x10=	$ 70
x15=	$	or	x30=	$-0-
+50=	$	or	+100=	$
Total	$	or	Total	$370

- [ ] Please charge my Deposit Account No. _____ the amount of $_____.
- [X] A duplicate copy of this sheet is enclosed.
- [X] A check in the amount of $ 370.00 to cover the filing fee is enclosed.
- [X] A check for $ 20.00 covering Recordation of Assignment fee is enclosed.
- [X] The Commissioner is hereby authorized to charge payment of the following fees associated with this communication or credit any overpayment to Deposit Account No. 13-4892. A duplicate copy of this sheet enclosed.
  - [X] Any additional filing fees required under 37 CFR 1.16.
  - [X] Any patent application processing fees under 37 CFR 1.17.
- [ ] The Commissioner is hereby authorized to charge payment of the following fees during the pendency of this application or credit any overpayment to Deposit Account No. _____. A duplicate copy of this sheet enclosed.
  - [ ] Any patent application processing fees under 37 CFR 1.17
  - [ ] The issue fee set in 37 CFR 1.18 at or before mailing of the Notice of Allowance, pursuant to 37 CFR 1.311(b).
  - [ ] Any filing fees under 37 CFR 1.16 for presentation of extra claims.

Respectfully submitted,

Dated: October 4, 1985

By: _____
Joseph E. Mueth
Registration No. 20,532

700 S. Flower St., Suite 2200
Los Angeles, CA 90017
(213) 688-7407

Attorney's Docket No. 107-145D-C **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Anticipated Classification of this application:

Class _____ Subclass _____

Prior application:

Examiner: ROSEN, S.

Art Unit: 1802

Box Patent Application
Commissioner of Patents and Trademarks
Washington, D.C. 20231

TRANSMITTAL OF FILING UNDER 37 CFR 1.60(b)

WARNING: *A C-I-P (continuation-in-part) cannot be filed under 37 CFR 1.60(b).*

WARNING: *A filing under 37 C.F.R. § 1.60(b) can only be made if the "prior application was a nonprovisional application and a complete application as set forth in § 1.51(a)(1)." 37 C.F.R. § 1.60(b)(1).*

WARNING: *Filing under 37 CFR 1.60 is permitted only if filed by the same or less than all the inventors named in the prior application. 37 CFR 1.60(b)(3).*

WARNING: *The filing of an application at the United States stage of an International Application requires an oath or declaration. 37 CFR 1.61(a)(4).*

WARNING: *The claims of this new application may be finally rejected in the first Office action where all claims of the new application are drawn to the same invention claimed in the earlier application and would have been properly finally rejected on the grounds or art of record in the next Office action if they had been entered in the earlier application. MPEP § 706.07(b).*

This is a request for filing a

☒ Continuation

☐ Divisional

application under 37 CFR 1.60, of pending prior application

Serial No. 07/ 303,712 filed on 1/27/89
(Date)

CERTIFICATION UNDER 37 CFR 1.10

I hereby certify that this 37 CFR 1.60 request and the documents referred to as attached therein are being deposited with the United States Postal Service on this date 3/9/98 in an envelope as "Express Mail Post Office to Addressee" service under 37 CFR 1.10, Mailing Label Number EI262826088US addressed to the: Commissioner of Patents and Trademarks, Washington, D.C. 20231.

Laura Velarde
(type or print name of person mailing paper)

Laura Velarde
(Signature of person mailing paper)

NOTE: *Each paper or fee filed by "Express Mail" must have the number of the "Express Mail" mailing label placed thereon prior to mailing. (37 CFR 1.10(b)).*

WARNING: *Certificate of mailing (first class) or facsimile transmission procedures of 37 CFR 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.*

of A. Said El Shami

(Inventor(s))

for METHOD FOR MEASUIRNG FREE LIGANDS IN BIOLOGICAL FLUIDS AND

(Title of invention)

ASSAY KITS FOR MEASUIRNG SAME

NOTE: 37 CFR 1.60 permits the omission of a declaration only if the prior application was complete as set forth in 37 CFR 1.51(a), namely, the prior application comprised at least (1) a specification, including a claim or claims; (2) a declaration; (3) drawings when necessary; and (4) the prescribed filing fee. Accordingly, as presently worded, 37 CFR 1.60 does not permit this procedure to be used where the prior application is pending but only the processing and retention fee required by 37 CFR 1.21(l) is paid or where the declaration was not filed.

1. Copy of Prior Application as Filed That is Attached

NOTE: Under 37 CFR 1.60, practice signing and execution of the application by the applicant may be omitted provided the copy is supplied by and accompanied by a statement by the applicant or his or her attorney or agent that the application papers comprise a true copy of the prior application as filed and that no amendments referred to in the declaration filed to complete the prior application introduced new matter therein.

NOTE: This statement need not be verified if made by an attorney registered to practice before the PTO. (37 CFR 1.60(b)).

☒ I hereby verify that the attached papers are a true copy of what is shown in my records to be the above identified prior application, including the oath or declaration originally filed. (37 CFR 1.60(b)(2))

The copy of the papers of prior application as filed which are attached are as follows:

☒ 29 page(s) of specification

☒ 4 page(s) of claims

☒ 1 page(s) of abstract

☒ 10 sheet(s) of drawing

(also complete part 6 below, if drawings are to be transferred)

☒ 2 pages of declaration and power of attorney

(If the copy of the declaration being filed does not show applicant's signature, because the attorney's records do not contain a copy of the signed declaration actually filed for the application, indicate thereon that it was signed and complete the following:)

☐ in accordance with the indication required by 37 CFR 60(b), my records reflect that the original signed declaration showing applicant's signature was filed on _____.

☐ the amendment referred to in the declaration filed to complete the prior application and I hereby state, in accordance with the requirements of 37 CFR 1.60(b), that this amendment did not introduce new matter therein.

2. Amendments

WARNING: "The claim of a new application may be finally rejected in the first Office action in those situations where (1) the new application is a continuing application of, or a substitute for, an earlier application, and (2) all the claims of the new application (a) are drawn to the same invention claimed in the earlier application, and (b) would have been properly finally rejected on the grounds or art of record in the next Office action if they had been entered in the earlier application." MPEP § 706.07(b).

☐ Cancel in this application original claims _____ of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

☒ A preliminary amendment is enclosed. (Claims added by this amendment have been properly numbered consecutively beginning with the number next following the highest numbered original claim in the prior application.)

NOTE: Only amendments reducing the number of claims or adding a reference to the prior application (§ 1.78(a)) will be entered before calculating the filing fee and granting the filing date. 37 CFR 1.60(b)(4).

NOTE: "When filing under Rule 1.60 retain at least one original claim from the patent application to assure a complete application." Notice of March 3, 1986 (1064 O.G. 37-38).

3. Petition for Suspension of Prosecution for the Time Necessary to File an Amendment

NOTE: Where it is possible that the claims on file will give rise to a first action final for this continuation application and for some reason an amendment cannot be filed promptly (e.g., experimental data is being gathered) it may be desirable to file a petition for suspension of prosecution for the time necessary).

(check the next item, if applicable)

☐ There is provided herewith a Petition To Suspend Prosecution For The Time Necessary to File An Amendment (New Application Filed Concurrently).

4. Information Disclosure Statement

(check this item, if applicable)

☐ An information disclosure statement is submitted herewith.

5. Fee Calculation (37 CFR 1.16)

CLAIMS AS FILED						
Number filed		Number Extra			Rate	Basic Fee 37 CFR 1.16(a) $730.00
Total Claims (37 CFR 1.16(c))	13	−20=	-0-	×	$ 22.00	-0-
Independent Claims (37 CFR 1.16(b))	2	−3=	-0-	×	$ 76.00	-0-
Multiple dependent claim(s), if any (37 CFR 1.16(d))				+	$240.00	

☐ Fee for extra claims is not being paid at this time. (37 CFR 1.16(d))

NOTE: If the fees for extra claims are not paid on filing they must be paid or the claims cancelled by amendment, prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency. 37 CFR 1.16(d).

Filing Fee Calculation $ 790.00

6. Small Entity Status

☐ A verified statement that this filing is by a small entity:

☐ is attached

☐ has been filed in the parent application and such status is still proper and desired (37 CFR 1.28(a))

Filing Fee Calculation (50% of above) $_____

NOTE: Any excess of the full fee paid will be refunded if a verified statement is filed within 2 months of the date of timely payment of a full fee then the excess fee paid will be refunded on request. 37 CFR 1.28(a).

NOTE: 37 CFR 1.28(a), last sentence states: "Applications filed under § 1.60 or § 1.62 of this part must include a reference to a verified statement in a parent application if status as a small entity is still proper and desired."

7. Drawings

☐ Drawings are enclosed

☐ formal

☐ informal

WARNING: DO NOT submit original drawings. A high quality copy of drawings should be supplied when filing a patent application. The drawings that are submitted to the Office must be on strong, white, smooth, and non-shiny paper and meet the standards of § 1.84. If corrections to the drawings are necessary, they should be made to the original drawings and a high-quality copy of the corrected original drawing then submitted to the Office. Only one copy is required or desired. Comments on proposed new 37 CFR 1.84. Notice of March 9, 1988 (1090 O.G. 57-62).

NOTE: "Identifying indicia, if provided, should include the application number or the title of the invention, inventor's name, docket number (if any), and the name and telephone number of a person to call if the Office is unable to match the drawings to the proper application. This information should be placed on the back of each sheet of drawing a minimum distance of 1.5 cm. (5/8 inch) down from the top of the page." 37 C.F.R. 1.84(c)).

8. Priority—35 U.S.C. 119

☐ Priority of application Serial No. 0 /_____ filed on _____ in _____ is claimed under 35 U.S.C. 119. (*country*)

☐ The certified copy has been filed in prior U.S. application Serial No. 0 /_____ on _____.

☐ The certified copy will follow.

9. Relate Back—35 U.S.C. 120

☒ Amend the specification by inserting, before the first line, the following sentence:

"This is a

☒ continuation

☐ divisional

of copending application(s)

☒ Serial number 07 /_303,712_____ filed on _1/27/89_____"

☐ International Application _____ filed on _____ and which designated the U.S."

NOTE: The proper reference to a prior filed PCT application which entered the U.S. national phase is the U.S. serial number and the filing date of the PCT application which designated the U.S.

10. Inventorship Statement

NOTE: "If the continuation or divisional application is filed by less than all the inventors named in the prior application a statement must accompany the application when filed requesting deletion of the names of the person or persons who are not inventors of the invention being claimed in the continuation or divisional application." 37 CFR 1.60(b)(4) [emphasis added].

(complete appropriate items (a) and (b))

(a) With respect to the prior copending U.S. application from which this application claims benefit under 35 USC 120 the inventor(s) in this application is (are):

(complete applicable item below)

☒ the same

☐ less than those named in the prior application and it is requested that the following inventor(s) identified above for the prior application be deleted:

_____

(type name(s) of inventor(s) to be deleted)

(b) The inventorship for all the claims in this application are

☒ the same

☐ not the same, and an explanation, including the ownership of the various claims at the time the last claimed invention was made, is submitted.

11. Assignment

☒ The prior application is assigned of record to
Diagnostic Products Corporation

☐ An assignment of the invention to _____

_____

is attached. A separate ☐ "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or ☐ FORM PTO 1595 is also attached.

NOTE: "If an assignment is submitted with a new application, send two separate letters - one for the application and one for the assignment." Notice of May 4, 1990 (1114 O.G. 77-78).

NOTE: When an assignee files a . . . divisional application (under . . . 1.60 . . .) reference may be made to a statement filed under 37 CFR 3.73(b) in the parent application, or a copy of that statement may be filed. Notice of April 30, 1993, 1150 O.G. 62-64.

12. Fee Payment Being Made At This Time

☐ Not Enclosed

☐ No filing fee is submitted. *(This and the surcharge required by 37 CFR 1.16(e) can be paid subsequently).*

☒ Enclosed

☒ basic filing fee $ 790.00

☐ recording assignment ($40.00; 37 CFR 1.21(h)) (See attached "COVER SHEET FOR ASSIGNMENT ACCOMPANYING NEW PATENT APPLICATION".)

☐ processing and retention fee ($130.00; 37 CFR 1.53(d) and 1.21(l)) $ _____

NOTE: 37 CFR 1.21(l) establishes a fee for processing and retaining any application which is abandoned for failing to complete the application pursuant to 37 CFR 1.53(d) and this, as well as the changes to 37 CFR 1.53 and 1.78 indicate that in order to obtain the benefit of a prior U.S. application, either the basic filing fee must be paid or else the processing and retention fee of § 1.21(l) must be paid within 1 year from notification under § 53(d).

Total fees enclosed $ 790.00

13. Method of Payment of Fees

☒ Enclosed is a check in the amount of $ 790.00

☐ Charge Account No. _____ in the amount of $_____. A duplicate of this request is attached.

NOTE: Fees should be itemized in such a manner that is clear for which purpose the fees are paid. 37 CFR 1.22(b).

14. Authorization To Charge Additional Fees

WARNING: *If no fees are being paid on filing do not complete this item.*

WARNING: *Accurately count claims, especially multiple dependent claims, to avoid unexpected high charges if extra claim charges are authorized.*

☒ The Commissioner is hereby authorized to charge the following additional fees which may be required by this paper and during the entire pendency of the application to Account No. 13-4892.

☒ 37 CFR 1.16 (a), (f) or (g) (filing fees)

☒ 37 CFR 1.16 (b), (c) and (d) (presentation of extra claims)

NOTE: Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must only be paid or these claims cancelled by amendment prior to the expiration of the time penod set for response by the PTO in any notice of fee deficiency (37 CFR 1.16(d)) it might be best not to authorize the PTO to charge additional claim fees, except possibly when dealing with amendments after final action.

☒ 37 CFR 1.17 (application processing fees)

WARNING: *While 37 CFR 1.17(a), (b), (c) and (d) deal with extensions of time under § 1.136(a) this authorization should be made only with the knowledge that: "Submission of the appropriate extension fee under 37 CFR 1.136(a) is to no avail unless a request or petition for extension is filed." [emphasis added]. Notice of November 5, 1985 (1060 O.G. 27).*

☐ 37 CFR 1.18 (issue fee at or before mailing Notice of Allowance, pursuant to 37 CFR 1.311(b)).

NOTE: Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance. 37 CFR 1.311(b)).

NOTE: 37 CFR 1.28(b) requires "Notification of any change in status resulting in loss of entitlement to small entity status must be filed in the application . . . prior to paying or at the time of paying . . . issue fee." From the wording of 37 CFR 1.28(b): (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity.

15. Power of Attorney

☒ The power of attorney in the prior application is to

Joseph E. Mueth, Esq. 20,532

(Attorney) *(Reg. No.)*

a. ☒ The power appears in the original papers in the prior application.

b. ☐ Since the power does not appear in the original papers, a copy of the power in the prior application is enclosed.

c. ☐ A new power has been executed and is attached.

d. ☒ Address all future communications to

(item d may only be completed by applicant, or attorney or agent of record)

Joseph E. Mueth, Esq.
225 South Lake Avenue, 8th Floor
Pasadena, CA 91101

16. Maintenance of Copendency of Prior Application

(this item must be completed and the papers filed in the prior application if the period set in the prior application has run.)

☐ A petition, fee and response has been filed to extend the term in the pending prior application until _____.

NOTE: The PTO finds it useful if a copy of the petition filed in the prior application extending the term for response is filed with the papers constituting the filing of the Continuation Application. Notice of November 5, 1985 (1060 O.G. 27).

☐ A copy of the petition for extension of time in the prior application is attached.

17. Conditional Petition for Extension of Time in Prior Application

(complete this item and file conditional petition in the prior application if previous item not applicable)

☐ A conditional petition for extension of time is being filed in the pending parent application.

NOTE: The PTO finds it useful if a copy of the petition filed in the prior application extending the term for response is filed with the paper constituting the filing of the continuation application. Notice of November 5, 1985 (1060 O.G. 27).

☐ A copy of the conditional petition for extension of time in the prior application is attached.

18. Abandonment of Prior Application (*if applicable*)

WARNING: *(Do not complete this item if the application being filed is a divisional of the prior application which is not being abandoned).*

NOTE: "A registered attorney or agent acting under the provisions of § 1.34(a), or of record, may also expressly abandon a prior application as of the filing date granted to a continuing application when filing such a continuing application." 37 CFR 1.138.

☒ Please abandon the prior application at a time while the prior application is pending or when the petition for extension of time or to revive in that application is granted and when this application is granted a filing date so as to make this application copending with said prior application.

19. Notification in Parent Application of the Filing of This Continuation Application

☐ A notification of the filing of this continuation is being filed in the parent application from which this application claims priority under 35 USC § 120.

20. Statement by Assignee (*if applicable*)

☒ In accordance with 37 CFR 3.73, I have reviewed the evidentiary documents establishing my/our ownership of the application identified herein, and certify that to the best of my/our knowledge and belief, title is with me/us who seek to take action.

☐ Assignment submitted herewith for recordal

I hereby declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Joseph E. Mueth
(type or print name of person signing declaration)

March 9, 1998
Date

Signature

225 South Lake Avenue, 8th Floor
(P.O. Address of Signatory)
Pasadena, CA 91101

☐ Inventor
☐ Assignee of complete interest
☐ Person authorized to sign on behalf of assignee
☒ Attorney or agent of record
☐ Filed under Rule 34(a)

Tel. No. :(626) 584-0396
Reg. No. 20,532
(if applicable)

(complete the following if applicable)

Diagnostic Products Corporation
(Type name of assignee)

5700 West 96th Street
(Address of assignee)
Los Angeles, CA 90045

Attorney of Record
(Title of person authorized to sign on behalf of assignee)

Assignment recorded in PTO on
10/4/85
Reel 4467
Frame 923

The statement under 37 CFR 3.73(b)

☒ has been filed in the parent application.

☐ a copy of the statement previously filed in the parent application is attached.

107-145D-C

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Group Art Unit: Not Assigned
A. Said El Shami	Examining Attorney: Not Assigned
Continuation Serial No.: Not Assigned (Divisional Serial No.: 07/303,712)	
	Date: March 9, 1998
Continuation Application Filed: Herewith (Divisional Filed 01/27/1989)	Pasadena, California
For: METHOD FOR MEASURING FREE LIGANDS IN BIOLOGICAL FLUIDS, AND ASSAY KITS FOR MEASURING SAME	

PRELIMINARY AMENDMENT

Hon. Commissioner of
Patent and Trademarks
Washington, D.C. 20231

Dear Sir:

Please amend the above-identified application as follows:

In the Drawings:

Please cancel the drawings originally filed in Divisional Application Serial Number 303,712 and use the most recent formal

drawings submitted by applicant in Divisional Application Serial Number 303,712.

In the Specification:

Page 5, line 14, cancel "analine" and insert --alanine--.

Page 5, line 18, cancel "analine" and insert --alanine--.

Page 12, line 10, cancel "succinimide" and insert --succinamide--.

Page 12, line 13, cancel "succinimide" and insert --succinamide--.

Page 11, line 10, cancel "carbonations" and insert --carbon atoms--.

Page 13, line 10, cancel "phyciological" and insert --physiological--.

Page 15, line 6 after Table 2, cancel "experiements" and insert --experiments--.

Page 18, line 6 of Table 8 cancel "0.0125" and insert --0.125--.

Page 20, line 5, cancel "resepective" and insert --respective--.

Page 21, line 3, cancel "A" and insert --At--.

Page 22, line 8 after Table 17, cancel "partically" and insert --partially--.

Page 22, line 4 from the bottom, cancel "reconsituted" and insert --reconstituted--.

Page 23, line 4, cancel "must" and insert --much--.

Page 33, line 6, cancel "succinimide" and insert --succinamide--.

Page 33, line 8, cancel "succinimide" and insert --succinamide--.

In the Claims:

Please cancel the claims and insert the following new claims:

Claim 35. A method for measuring the concentration of free thyroxine or triiodothyronine ligands in biological fluids in the presence of bound ligand and endogenous binding proteins, including albumin, without disturbing the equilibrium between the free ligand and the protein bound ligand, comprised of the following steps:

(a) incubating a sample of the biological fluid with (i) a ligand analog tracer which, due to its chemical structure, does not bind to some of the endogenous binding proteins, (ii) a specific ligand binder at a concentration which does not significantly strip bound ligand from said endogenous proteins and having an affinity constant from about 0.246×10^5 up to about 5×10^5 l/mol and, (iii) at least one specific chemical inhibitor reagent that inhibits the binding of the ligand analog tracer to other endogenous binding proteins, said specific chemical inhibitor reagent being present in a concentration sufficient to displace the ligand analog tracer from at least one other endogenous binding protein without

displacing the native ligand form said endogenous binding proteins;

(b) separating the ligand analog tracer bound to the specific binder from unbound tracer; and

(c) determining the concentration of free ligand in said biological fluid.

Claim 36. A method for measuring the concentration of free thyroxine or triiodothyronine free ligands in biological fluids in the presence of bound ligand and endogenous binding proteins, including albumin, without disturbing the equilibrium between the free ligand and the protein bound ligand, comprised of the following steps:

(a) incubating a sample of biological fluid with (i) a ligand analog tracer which, due to its chemical structure, does not bind to some of the endogenous binding proteins, (ii) a specific ligand binder at a concentration which does not significantly strip bound ligand from said endogenous proteins and having an affinity constant from about 0.246×10^5 up to about 5×10^5 l/mol and, (iii) specific chemical inhibitor reagents that alone or in combination inhibit the binding of the ligand analog tracer to other endogenous binding proteins, said specific chemical inhibitor reagents being present in a concentration sufficient to displace the ligand analog tracer from at least one other endogenous binding protein without

displacing the native ligand from said endogenous binding proteins;

(b) separating the ligand analog tracer bound to the specific binder from unbound tracer; and

(c) comparing the bound fraction in said sample to the bound fraction of a given set of known free ligand calibrators to determine the concentration of free ligand in said biological fluid.

Claim 37. The method of claim 35 wherein the chemical inhibitor agent is 2,4-dinitrophenol at a concentration of 5-10 mmol/l.

Claim 38. The method of claim 35 wherein the chemical inhibitor agent is sodium salicylate at a concentration of 40-125 mmol/l.

Claim 39. The method of claim 35 wherein the chemical inhibitor reagent is sulfobromophthalein at a concentration of 0.8 x 10^{-5} M to 1.6 x 10^{-5} M.

Claim 40. The method of claim 35 wherein the chemical inhibitor reagent is oleic acid at a concentration of 0.4-0.8 mmol/l.

Claim 41. The method according to claim 35 or 36 wherein the specific ligand binder is an antibody to said free ligand.

Claim 42. The method according to claim 35 or 36 wherein the specific ligand binder is immobilized on a solid substrate.

Claim 43. A method according to claim 42 wherein the solid substrate is polypropylene.

Claim 44. The method according to claims 35 to 36 wherein the ligand analog tracer is labelled with at least one radioactive atom, an enzyme, fluorophor, light chromophore or chemiluminescent group.

Claim 45. The method according to claim 44 wherein the ligand analog tracer is N-^{125}I-L-triiodothyronine succinimide or N-^{125}I-L-thyroxine succinimide.

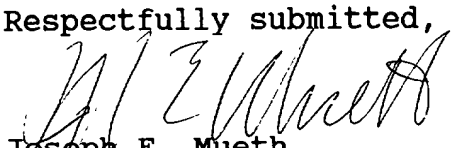
Claim 46. The method according to claims 35 or 36 when carried out at about 37°C and at about pH 7.4.

Claim 48. The method according to claim 36 wherein said free ligand calibrators have been prepared by adding different amounts of the ligand to ligand-free human serum, calibrating by equilibrium dialysis and assigning free ligand values.

The above amendments are required to correct obvious typographical errors.

Entry of this Amendment is hereby requested.

Respectfully submitted,



Joseph E. Mueth
Registration No. 20,532

Date: March 9, 1998

225 South Lake Avenue
8th Floor
Pasadena, CA 91101
Telephone: (626) 584-0396

DIVISION-CONTINUATION PROGRAM APPLICATION TRANSMITTAL FORM	ATTORNEY'S DOCKET NO. 107-145D

DOCKET NUMBER	ANTICIPATED CLASSIFICATION OF THIS APPLICATION: CLASS	SUBCLASS	PRIOR APPLICATION EXAMINER	ART UNIT
107-145D			J. Spiegel	128

To the Commissioner of Patents and Trademarks:

This is a request for filing a ☐ continuation ☒ divisional application under 37 CFR 1.60, of pending prior a application serial no. 784,857 filed on October 4 19 85, of A. Said El Shami for METHOD FOR MEASURING FREE LIGANDS IN BIOLOGICAL FLUIDS AND ASSAY KITS FOR MEASURING SAME.

1. Enclosed is a copy of the latest inventor signed prior application, including the oath or declaration as originally filed. I hereby verify that the attached papers are a true copy of the latest inventor signed prior application serial no. 784,857 as originally filed on October 4 19 85, and further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and fruther that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Claims	(1) For	(2) Number filed	(3) Number extra	(4) Rate	(5) Calculations
	Total Claims	9 − 20=	0	X $12.00	$
	Independent Claims	2 − 3=	0	X $34.00	
	Multiple Dependent Claim(s) (if applicable)			+$110.00	
			Basic Fee		+ $340.00
			Total of above Caculations =		
	Reduction by 1/2 for filing by small entity (Note 37 CFR 1.9, 1.27, 1.28) if applicable, affidavit must be filed also.				−
			Total National Fee		$ 340.00

2. ☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. 13-4892. A duplicate copy of this sheet is enclosed.

3. ☐ A check in the amount of $ 340.00 is enclosed.

4. ☒ Cancel in this application original claims 2 - 27 of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

5. ☒ Amend the specification by inserting before the first line the sentence: This application is a ☐ continuation, ☒ division, of application serial no. 784,857, filed Oct. 4,1985.

6. ☐ Transfer the drawings from the pending prior application to this application and abandon said prior application as of the filing date accorded this application. A duplicate copy of this sheet is enclosed for filing in prior application file. (May only be used if signed by person authorized by § 1.138 and before payment of issue fee.)

(continued)

a. ☒ New formal drawings are enclosed.

b. ☐ Priority of application serial no. _____ filed on _____ in

_____ is claimed under 35 U.S.C. 119.
(country)

☐ The certified copy has been filed in prior application serial no. _____

filed _____ .

7. ☒ The prior application is assigned of record to Diagnostic PRODUCTS Corporation .

8. ☒ A preliminary amendment is enclosed.

9. ☐ A verified statement claiming small entity status is enclosed in parent application Serial Number _____ , filed _____ and is still proper.

10. ☐ Also enclosed _____

11. ☒ The power of attorney in the prior application is to

Joseph E. Mueth, REgistration No. 20,532

a. ☒ The power appears in the original papers in the prior application.

b. ☐ Since the power does not appear in the original papers, a copy of the power in the prior application is enclosed.

c. ☒ Address all future communications : (May only be completed by applicant, or attorney or agent of record)

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1/22/89 (date) _____ (signature)

Address of signator: ☐ inventor(s) ☐ filed under § 1.34(a)
☐ assignee of complete interest
☒ attorney or agent of record

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I hereby certify that this correspondence is being deposited with the United States Postal Service as Express Mail (Label No. B34445114) in an envelope addressed to Commissioner of Patents and Trademarks, Washington, D.C. 20231, on October 4, 1985.

Dated: October 4, 1985

Suzi McCraw

For Joseph E. Mueth
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METHOD FOR MEASURING FREE LIGANDS IN BIOLOGICAL FLUIDS, AND ASSAY KITS FOR MEASURING SAME

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BACKGROUND OF THE INVENTION

For several decades equilibrium dialysis techniques were the only available method for the measurement of free hormones in serum, and until recently were the only methods considered reliable. Equilibrium dialysis methods in this context suffer from several drawbacks including poor precision. tediousness and so on; but above all their results are highly dependent on the purity of the tracers used.

Ellis and Ekins, R. (Acta Endocr. (KbH.) Suppl. 177:106, 1973), disclosed a direct method for free hormone determinations in their paper "Direct Measurement By Radioimmunoassay of the Free Thyroid Hormone Concentration in Serium." This represented a major improvement over equilibrium dialysis methods because it allowed for the direct measurement by radioimmunoassay (RIA) of free ligand levels in serum dialysates, thus circumventing the problem of tracer purity. This method is now considered by many as the reference methodology for free hormone measurements. It is, however, still time consuming and operator-dependent, and it is unavailable to most small laboratories.

Indirect methods for the estimation of free hormone concentrations which were introduced shortly thereafter include the testosterone/steroid hormone binding globulin (SHBG) ratio, the thyroxine (T4)/thyroid binding globulin (TBG) ratio, the free T4 index (based on the product of triiodothyronine (T3) uptake and T4), and the free androgen index.

Ekins, R. (Free Thyroid Hormones; Proceedings of the International Symposium held in Venice, December 1978, Amsterdam: Excerpta Medica, 1979 72-92), introduced the concept of "direct

dynamic methods" in which an anti-free ligand antibody is used in direct contact with the biological fluid during dialysis. This constitutes the basis for so-called "immunoextraction" methods.

One such method is taught in U.S. Patent No. 4,046,870 in which a two-tube immunoassay method measures the rate of transfer of T4 from binding proteins to T4-specific antibody. This method suffered from several analytical and clinical shortcomings which made it virtually just another free T4 index assay.

A second method, introduced by Clinical Assays (Cambridge, MA 02139), was a true immunoextraction method. It used a single-tube, two-stage, sequential (back-titration) technique. In this method, a serum sample is incubated with immobilized antibody; then, following a wash step, unoccupied sites on the immobilized antibody are "back-titrated" using labeled ligand. In this approach, the serum is never in contact with the labeled ligand. Although theoretically sound, it suffers from poor sensitivity and precision, and both reactions require exact timing.

Single-step immunoextraction methods for the determination of free ligand concentrations in biological specimens were the obvious next step in the development of free ligand assay systems. These methods rely on chemical rather than physical separation of labeled ligand from endogenous binders. In order to achieve this objective, several approaches can be adopted, as detailed below.

The prior art discloses that by chemically altering the structure of a given ligand, its binding to endogenous binders is reduced or diminished. This has been amply demonstrated

for steroid hormones. (See the discussion of free testosterone below.) In the case of thyroid hormones, Ross, J.E. and Tapley, D.F. (Effect of various analogues on the binding of labeled thyroxine to thyroxine-binding globulin and prealbumin, Endocrinology 79:493, 1966), have shown that the binding of TBG (thyroid binding globulin) to T4 is inhibited if a fairly bulky substitution is made at the 3' position of the T4 molecule. In addition, Schall, R.F., et al (An enzyme-labeled immunoassay for the measurement of unsaturated thyroid hormone binding capacity in serum and plasma, Clin. Chem. 25:1078 (abstract) 1979), and Kleinhammer, G., et al (Enzyme immunoassay for determination of thyroxine binding index, Clin. Chem. 24:1033, 1978), independently demonstrated that TBG fails to bind to conjugates formed by labeling T4 with horseradish peroxidase. This fact constitutes the basis for the single-step immunoextraction method described in U.S. Patent No. 4,410,633 to Corning Glass Works, for the measurement of free thyroxine and free 3,5,3'-triiodothyronine wherein horseradish peroxidase is chemically attached to T4 and T3 and later radiolabeled.

In addition, the prior art also discloses that T3 and T4 require the following molecular structure for maximal binding to endogenous binding proteins, viz. TBG, thyroid binding pre-albumin (TBPA), albumin, Snyder, S.M, et al (Binding of thyroid hormones and their analogues to thyroxine-globulin in human serum, J. Biol. Chem. 251:6489, 1976); Sterling, K., et al (Equilibrium dialysis studies of the binding of thyroxine by human serum albumin, J. Clin. Invest. 41:1021, 1962):

1. The L-alanine side chain configuration;

2. The presence of 4'-hydroxyl group (primarily for TBPA and albumin binding); and

3. The presence of two (halogen) substituents in the inner and outer rings (positions 3,5,3' and 5').

Several hundred T3 and T4 analogs have been synthesized and studied for their ability to bind to thyroid hormone binding proteins.

U.S. Patent No. 4,366,143 and its European counterpart, Patent No. 00 26 103, broadly describe the use of such analogs as tracers in a single immunoextraction using simultaneous rather than sequential titration of antibody for the measurement of free hormones. (For convenience, these patents will be collectively referred to hereinafter as the "Amersham" patent.)

An intact analine side chain is required for optimal binding of T4 and T3 to TBG: the amino group on the analine side chain is the essential constituent. Analogs described in the Amersham patent are T3 and T4 molecules modified at the analine side chain. Although theoretically these analogs do not bind TBG to any significant extent, they undoubtedly bind albumin and TBPA significantly since the 4'-hydroxyl group on the T3 and the T4 molecules is left intact. It is well established that the binding of albumin and TBPA to the thyronines is quantitative, especially under physiological conditions, Sterling, K. (Molecular structure of thyroxine in relation to its binding by human serum albumin, J.Clin Invest. 43:1721, 1964), and Pages, et al (Binding of thyroxine and thyroxine analogs to human serum prealbumin, Biochem 12:2773, 1973).

The failure of the Amersham patent to recognize the importance of albumin and TBPA binding to the thyronines

renders the patent's teachings inadequate for the true measurement of free T3 and free T4 in biological fluids. In fact the commercially available reagents based on the patent yield misleading and inaccurate free hormone results. This is particularly true in several pathological conditions characterized by significant alterations in the circulating albumin level.

Recent literature has shown that the albumin concentration correlates directly with free T4 concentrations generated by the Amersham assay system. In addition, it is well documented that Amersham's method consistently yields falsely decreased free T4 results in third-trimester pregnancies and in patients suffering from severe non-thyroidal illness, while yielding falsely elevated free T4 levels in cases of familial dysalbuminemic hyperthyroxinemia, a condition in which T4 is abnormally bound to circulating albumin.

During pregnancy, albumin circulates at lower than normal levels, especially during the third trimester. Since Amersham's labeled analog T4 tracer binds albumin and TBPA to a significant extent (greater than 99%), one would expect the Amersham assay system to yield lower than normal free T4 results during the third trimester: more analog tracer is available to bind T4 antibody, resulting in higher binding and lower apparent dose.

Non-esterified free fatty acids are capable of displacing labeled analog from albumin; moreover, they circulate at higher than normal concentrations during pregnancy. This could explain the lower than expected free T4 values encountered during pregnancy when assayed by the Amersham method; apparent free T4 levels would be significantly lower than expected if albumin binding to the labeled analog is substantial.

This situation is also well documented in cases of heparin therapy, where a significant elevation of non-esterified free fatty acids is present. Free T4 and free T3 levels when measured by Amersham's method on heparin-treated patients show lower than normal levels.

The same problem occurs for non-thyroidal illness, where free T3 and T4 values generated by the Amersham method have been shown to be significantly lower than for a euthyroid population, when compared to a direct equilibrium dialysis method.

The Amersham patent procedure has been found wanting by workers in the art as manifested by the observance of false and erroneous measurements of free ligand levels. Applicant has discovered that the problem stems from binding of the ligand analog tracer to certain endogenous proteins, e.g., albumin in biological fluids. I have discovered that this problem can be overcome by the use of specific chemical inhibitor reagents. This discovery represents a major advance in the art and it is believed to be deserving of a patent.

SUMMARY OF THE INVENTION

Briefly, this invention comprises a method for measuring free ligands in biological fluids in the presence of bound ligand and endogenous binding proteins, without disturbing the equilibrium between the free ligand and the protein-bound ligand, comprised of the following steps: (a) incubating a sample of biological fluid with (i) a ligand analog tracer which, due to its chemical structure, does not bind to some of the endogenous binding proteins, (ii) a specific ligand binder and (iii) at least one specific chemical inhibitor reagent that inhibits the binding of the ligand analog tracer to other endogenous binding proteins; (b) separating the ligand analog tracer bound to the specific binder from unbound tracer; and (c) determining the concentration of free ligand in said biological fluid.

It is an object of this invention to provide a new and improved method for measuring free ligands in biological fluids.

More particularly, the present invention has as its object the truer measurement of free ligands in biological fluids.

These and other objects and advantages of my invention will be apparent from the detailed description which follows.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

The present invention addresses the deficiencies encountered in the Amersham patent and effectively corrects for the inconsistencies in free thyroid results generated by Amersham's analog method.

The present invention uses labeled analogs for T3 and T4 that are modified at the analine side chain. Specifically, the α-amino group is modified to prevent their binding to TBG. Meanwhile, steps have been taken to prevent such labeled analogs from binding to albumin and TBPA. This is accomplished by carefully selecting an exogenous chemical reagent or reagents that alone or in combination are able to bind to unoccupied binding sites on the albumin and TBPA molecules, thus saturating these binding proteins and effectively eliminating their capacity to bind to thyronine analogs and to other endogenous substances such as non-esterified free fatty acids. These exogenous chemicals should not bind to TBG and their concentration should be such as not to displace any bound hormone from albumin or TBPA.

The association constant for albumin and T4 is approximately 500,000. (This estimate is based on the assumption that the number of binding sites on the albumin molecule available for thyroxine is equal to 1, and that the apparent association constant in liters per mole - i.e. the equilibrium constant in the direction of complex formation - is 5×10^5.) Likewise, the association constant for albumin and T3 is approximately 24,600. It is well established that albumin has a higher affinity for free T3 and T4 and their analogs than for anionic dyes, but a much higher affinity for free fatty acids than T3 and T4 and their analogs.

Albumin has a relatively low association constant for single aromatic compounds; the highest association constants are for 2,4-dinitrophenol (11,000) and salicylate (2,800).

In order to maintain strict equilibrium conditions in vitro during the immunoextraction reaction one has to maintain strict physiological conditions; this entails the use of pH = 7.4. At that pH, thyronine molecules have three charged groups: the anionic carboxylate ion, the cationic α-amino group and the anionic phenolate ion. (The latter is 82% ionized.) The presence of albumin or TBPA under these physiological conditions yields a highly charged albumin with a relatively large number of cationic amino groups. These cationic amino groups on the albumin molecule bind the anionic phenolate ion on the thyronine molecules. Such an interaction is the main cause of albumin binding to the labeled analog in both the Amersham patent method and the Corning patent method.

The present invention makes use of the fact that 2,4-dinitrophenol (DNP) and sodium salicylate with their relatively high association constants to albumin and TBPA will also be ionized and charged under these physiological conditions of pH, yielding charged anionic phenolate ion capable of interaction with the charges on the albumin and TBPA molecules. When either 2,4-dinitrophenol or sodium salicylate or both are present in excess, the binding of labeled T3 and T4 analogs to albumin and TBPA is virtually eliminated. This method of blocking albumin and TBPA by appropriate concentrations of 2,4-dinitrophenol and/or sodium salicylate is an effective means for eliminating the erroneous assay results caused by albumin in free thyroid hormone immunoex- traction analog methods.

The present invention is applicable to a variety of other chemical inhibitor reagents, that is, reagents capable of blocking unwanted reaction of the ligand analog tracer to circulating endogenous binding proteins. The substituted monoaryl organic compounds are exemplary. The substituents on such compounds include nitro, carboxyl, carboxyl salts and the like. The monoaryl compounds have a phenolic hydroxyl group which are particularly useful. Another suitable category are the dyes such as sulfobromophthalein, orange red, bromocresyl blue and the like. The higher (over about 5 carbonations) fatty acids such as oleic acid are also useful. Still other compounds will be apparent to those skilled in the art. For example, many amino acids have a high affinity to albumin and hence are useful in the practice of this invention, e.g., tryptophan. Another suitable category are T3, T4 or testosterone analogs which displace labeled analog from endogenous proteins while not binding to the antibody or other specific ligand binder.

This invention can be used to detect the concentration of any of the free ligands normally found in human body fluid. For example, the free ligand can be thyroxine, tri-iodothyroxine, testosterone, cortisol, progesterone, oestradiol, hormones and steroids generally, also drugs and products of drug metabolism, vitamins such as B12, toxins, and the like.

In general, specific ligand binder is one which couples or binds to the free ligand and it may be a specific antibody for the free ligand or other binding agent. In general, the specific ligand binders appropriate to the various free ligands are known and need not be further described.

The ligand analog tracer is labeled in some way so as to be detectable or observable. Radiolabels are well-known and applicable, as are the other labeling means previously

employed in this art, including enzymes, fluorophors, chromophores and chemiluminescent groups integral with the ligand analog tracer molecule.

Free Thyroid Hormones

Antibodies to both L-thyroxine and 3,5,3'-triiodothyronine were produced in rabbits by well-established, conventional techniques using bovine serum albumin-T4 and -T3 as the immunogens.

Analogs of diiodothyronine (T2) and T3 were prepared by succinylating the α-amino group on the analine side chain to produce N-L-diiodothyronine succinimide and N-L-triiodothyronine, respectively, which were then iodinated by conventional iodination procedures to produce, respectively, N-^{125}I-L-triiodothyronine succinimide and N-^{125}I-L-thyroxine succinimide. The tracers were then compounded in 0.01M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer, pH 7.4 and 0.01% sodium azide. 0.1% charcoal-absorbed human serum albumin (CAHSA) free of any apparent T3 or T4, and blocking agents were added as described in specific examples below. Different amounts of T3 or T4 were added to human serum free of any apparent T3 and/or T4, calibrated in terms of direct equilibrium dialysis, and assigned values for each level.

T3 and T4 antibodies were immobilized on the inner walls of polypropylene 12X75mm tubes by passive adsorption as described in Catt, K., et al (Solid phase radioimmuoassay in antibody-coated tubes, Science 158:1570, 1967).

For the assay of free T4, 50 µl of calibrator or patient sample is pipetted into anti-T4 antibody-coated tubes, followed by 1.0 ml of the labeled T4 analog. The tubes are then incubated for 60 minutes at 37°C. After this incubation the tubes are decanted and the bound radioactivity is counted. Results are calculated from the calibration curve and expressed in ng/dl.

For free T3 assay, 100 µl of calibrator or patient sample is pipetted into anti-T3 antibody-coated tubes, followed by 1.0 ml of labeled T3 analog tracer. The tubes are incubated for three hours at 37°C, then decanted and radioactivity counted. The results are calculated as for free T4 and expressed in pg/ml.

Free Thyroid Hormone Examples

Example 1: The choice of antibodies for the free T3 and free T4 assay systems was determined by the fact that the free hormone is in phyciological equilibrium with its transport proteins. This equilibrium should be maintained when an antibody directed against the hormone is added to the system. It is essential to select an antibody which is appropriate in terms of its affinity constant and its specificity for the free analyte. Such antibodies should also have slow reaction kinetics.

For free thyroxine (T4) an antibody with a working titer or dilution of 1:250,000 was selected (2.0 ng IgG/tube). In order to check the effect of tracer binding to the antibody in the presence and absence of albumin and albumin-blocking agents, antibody-coated tubes were prepared using titers of 1:250,000 (2.0 ng IgG/tube) and 1:25,000 (20.0 ng IgG/tube). Maximum bindings were determined following the free T4 protocol described above. The results are tabulated below in table 1.

Table 1. Free T4.

	B T		
	[illegible]	[illegible]	
[illegible] A	[illegible]	[illegible]	without CAHSA [illegible] albumin
[illegible] B	[illegible]	[illegible]	without CAHSA [illegible] albumin [illegible]
[illegible] C	[illegible]	[illegible]	with [illegible]
[illegible] D	[illegible]	[illegible]	with [illegible]
[illegible] E	[illegible]	[illegible]	with [illegible]
[illegible] F	[illegible]	[illegible]	with [illegible]
[illegible] G	[illegible]	[illegible]	with [illegible]
[illegible] H	[illegible]	[illegible]	with [illegible]

In the absence of albumin or any other protein, the binding of the analog ^{125}I-T4 tracer to antibody at both antibody titers is of equal magnitude. In the presence of albumin-2 mg albumin/tube, contributed jointly by the tracer and the zero calibrator-the analog tracer does not bind to the higher titer antibody, while binding to the lower titer antibody at only 9.4% (tracer D). In the presence of only 1 mg albumin/tube, the binding of tracers B and C to the high titer antibody is negligible-2.6% and 1.4%, respectively -whereas binding to the lower titer antibody is significant - 18.1% and 15.0%, respectively.

The following conclusions can be drawn from the results of these experiments:

1. Albumin at concentrations of 1 to 2 mg/tube substantially binds to the tracer analog in the presence of 2.0 ng IgG antibody/tube.

2. 2.0 ng IgG antibody/tube has a lower affinity than albumin for the analog tracer.

3. In the presence of albumin blocking agents, the binding of labeled T4 analog to the antibody is restored.

The same experiments were also conducted for the free T3 assays. The tabulated results support similar conclusions (Table 2).

Table 2. Free T3.

Ab Titer:	% B/T 1:9,000	1:90,000	
Tracer A	70.6%	46.3%	without CAHSA; no zero calibrator (system devoid of albumin)
Tracer B	6.0%	1.0%	without CAHSA; with zero calibrator (100 µl)
Tracer C	6.3%	1.2%	with 1.0 mg/ml CAHSA tube; no zero calibrator
Tracer D	5.4%	0.9%	with 1.0 mg/ml CAHSA/tube + 100 µl zero calibrator
Tracer E	42.9%	22.5%	with 1.0 mg/ml CAHSA/tube + 100 µl zero calibrator + 1.0 mg/ml Na salicylate
Tracer F	59.2%	35.0%	with 1.0 mg/ml CAHSA/tube + 100 µl zero calibrator + 5 mg/ml Na salicylate
Tracer G	46.0%	23.1%	with 1.0 mg ml CAHSA tube + 100 µl zero calibrator + 1.0 mg/ml Na salicylate + 1.0 mg ml 2,4-dinitrophenol
Tracer H	37.7%	28.5%	with 1.0 mg/ml CAHSA/tube + 100 µl zero calibrator + 25 mg ml Na salicylate + 0.15 mg ml 2,4-dinitrophenol

Thus, the concentration of the antibody used in a free hormone assay is critical, and must be carefully adjusted so as not to displace bound analyte from endogenous proteins. The teachings of the Amersham and Corning patents do not disclose the concentrations of the antibodies used to measure free T3 and free T4. However, based on the experiements summarized above, it can be assumed that both the Amersham and the Corning patents must have used substantially higher antibody concentrations in order to bring about reasonable binding between the antibody and the analog tracer, since neither patent employs blocking agents.

Example 2: The working antibody concentrations established on the basis of Example 1 above are 5.5 and 2.0 ng IgG/tube of T3 antibody and T4 antibody, respectively. In order to

determine the appropriate albumin blocking agent or agents for use in the free T3 and free T4 assay systems, the following compounds were added to the analog tracers in the concentrations specified. (Each tracer also contained 1 mg/ml of charcoal absorbed human serum albumin.) Maximum binding was determined for each tracer. The zero calibrator was also added to each set of maximum binding tubes.

It must be emphasized that the scope of this invention is not limited to the examples used in Tables 3 through 11. They are presented here to show that, at the antibody concentrations selected, binding of the analog tracers will increase with increasing amounts of albumin blocking reagents added, until it reaches a plateau. This also shows that binding of the T3 and T4 labeled analog is eliminated by the use of an appropriate concentration of specific albumin blocking agents.

Table 3. Free T3.

2,4 dinitrophenol	B/T
10 ug/ml	1.0 %
50	1.4 %
100	2.9
150	4.3 %
200	5.8 %
400	10.0 %
800	16.8 %
1000	17.2 %
3000	28.2 %
3500	27.5 %
4000	27.3 %

Table 4. Free T3.

oleic acid	% B/T
0.0125 mmol/l	1.2
0.025	1.3 %
0.05	1.3
0.125	1.7 %
0.25	3.5 %
0.375	12.5 %
0.50	17.6 %
0.75	19.0 %
1.0	15.5 %

Table 5. Free T3.

sodium salicylate	% B T
0.25 mg ml	8.8
0.50	14.0
1.0	21.3
2.0	26.7*
5.0	35.8
10.0	36.7
20.0	33.3
25.0	30.4*
30.0	27.8

Table 6. Free T3.

sodium salicylate	2,4 dinitrophenol	% B T
1.0 mg ml	1.0 mg ml	20.3
5.0	1.0	29.8
5.0	5.0	32.5
5.0	0.1	34.2
5.0	0.15	38.2
15.0	0.15	33.0
25.0	0.15	29.5

Table 7. Free T4.

2,4-dinitrophenol	% B T
10 µg ml	2.4
50	4.3
100	8.0
150	11.0
200	13.3
400	22.9
800	33.8
1,000	34.4
2,000	43.2
2500	45.5

Table 8. Free T4.

oleic acid	B T
0.00625 mmol l	1.7
0.0125	1.7%
0.025	1.9%
0.0625	2.3%
0.0125	3.5%
0.1875	6.8%
0.25	14.3 %
0.375	30.5%
0.50	32.7
0.75	32.9%
1.00	31.0

Table 9. Free T4.

sodium salicylate	B T
0.05 mg ml	3.3 %
0.075	7.2
0.10	8.5%
0.15	11.7
0.25	15.6 %
0.50	22.0%
1.0	28.3
2.0	37.3 %
3.0	43.0
4.0	45.4 %
20.0	45.0
25.0	40.0
30.0	40.0

Table 10. Free T4.

sodium salicylate	2 - [illegible]	B T
1.0 mg ml	[illegible]	[illegible]
5.0	[illegible]	[illegible]
5.0	[illegible]	[illegible]
5.0	[illegible]	18.2
5.0	[illegible]	[illegible]
10.0	[illegible]	[illegible]
25.0	[illegible]	[illegible]

Example 3: The following experiment was designed to demonstrate that albumin has no effect on the free T3 and free T4 assay systems.

Ten samples - 5 from normal individuals and 5 from females in the third trimester of pregnancy - were each divided into 4 aliquots. To three of these aliquots, lyophilized charcoal-absorbed human serum albumin was added in concentrations of 10, 20 and 50 mg/ml. The four aliquots were then processed in duplicate, as described above, in free T3 and free T4 assays using four different tracers. The mean value for each albumin concentration (N = 5) was then plotted for each tracer (Figures 1 to 16). For free T3 and free T4, the tracers are as follows:

Table 11. Free T4 Tracers.

Tracer I	contains 0.5 mg ml sodium salicylate
Tracer II	contains 1 mg ml sodium salicylate and 1 mg ml 2,4-dinitrophenol
Tracer III	contains 5 mg ml sodium salicylate
Tracer IV	contains 25 mg ml sodium salicylate and 0.15 mg ml 2,4 dinitrophenol

Table 12. Free T3 Tracers.

Tracer I	contains 1 mg ml sodium salicylate
Tracer II	contains 1 mg ml sodium salicylate and 1 mg ml 2,4-dinitrophenol
Tracer III	contains 5 mg ml sodium salicylate
Tracer IV	contains 25 mg ml sodium salicylate and 0.15 mg ml 2,4 dinitrophenol

It is evident from the outcomes of these experiments that results generated by tracer IV for both free T3 and free T4 are unaffected by the addition of albumin up to 5.0 gm/dl, for an approximate total albumin concentration of 8.0 gm/dl.

Example 4: In order to determine whether thyroid binding globulin (TBG) will bind the labeled free T3 and free T4 analog tracers, the following experiment was conducted using tracer IV from Example 3. TBG resin stripped of all apparent T4 and T3 was added to the resepective zero calibrator for each free T4 and free T3 assay in the concentrations specified below. The observed percent bound (B/B_O) values are shown in the Table.

Table 13.

	B B	
	FT4	FT3
zero cal	100%	100%
+ 10 mg/ml	95%	99%
+ 20 mg ml	96%	99%
+ 50 mg/ml	94%	95%

Example 4a: This experiment was designed to check the effect of adding albumin to the zero calibrator using tracer IV from Example 3. Human serum albumin was charcoal-absorbed to remove any apparent T3 and T4 and was added to the respective zero calibrator for each free T3 and T4 in the concentrations indicated. Again, percent bound values were checked.

Table 14.

	B B_o	
	FT4	FT3
zero cal	100%	100%
+ 10 mg ml	97%	97%
+ 20 mg ml	98%	100%
+ 50 mg ml	95%	95%

It is obvious from Examples 4 and 4a that neither albumin nor TBG binds the analog tracers under the conditions specified.

Example 5: A high concentrations, sulfobromophthalein - a dye capable of binding to albumin - is able to displace T3 and T4 from the albumin molecule. Sulfobromophthalein at low concentrations is ineffective in blocking T3 and T4 analog tracers from binding to albumin. Iodinated T4 analog was compounded as described above and divided into five aliquots. To each aliquot the following reagents were added.

Table 15.

Tracer 1	25 mg ml sodium salicylate + 0.15 mg ml 2,4-dinitrophenol (w/v)
Tracer 2	0.25 mg ml sulfobromophthalein
Tracer 3	0.5 mg ml sulfobromophthalein
Tracer 4	1.0 mg ml sulfobromophthalein
Tracer 5	1.0 mmol l oleic acid

Each tracer was used in a separate assay for the measurement of free T4 in 20 samples under identical experimental conditions.

Considering tracer 1 as the reference and comparing the others to it, the following results were obtained.

Table 16.

	Tracer 1	Tracer 2	Tracer 3	Tracer 4	Tracer 5
Total CPM	56 145	59 182	58 391	55 886	60 030
NSB	2.5 %	0.6	0.5	0.7	0.5
MB	38 %	28.7	28.0	27.5	30.2
r*	−1.00075	−0.99864	−0.99975	−0.99975	−0.99936
Calibration Range B/B (1.1 – 90.0) ng/dl	93.3 – 8.5	93.4 – 7.4	93.1 – 4.6	93.6 – 1.1	92.8 – 3.5
Intercepts ng/dl					
20	2.0	1.8	2.1	2.1	1.8
50	1.8	0.2	0.3	0.3	0.2
SE	0.12	0.04	0.03	0.2	0.04
MB (0.2 ng/dl samples)					
95 %	1.6	0.6	1.2	1.7	0.7

*Correlation coefficient (an index of linearity)

Table 17. Regressions

Tracer 2 =	[illegible] Tracer 1 − 1.17	r =	[illegible]
Tracer 3 =	0.81 Tracer 1 − 0.14	r =	0.945
Tracer 4 =	1.52 Tracer 1 − 0.36	r =	0.956
Tracer 5 =	0.11 Tracer 1 − 0.51	r =	0.268

The results of using tracer 3 with 0.05% sulfobromophthalein correlate significantly with those obtained using tracer 1. Results generated using tracer 3 are, however, approximately 20% lower than those generated using tracer 1. Although tracer 4 correlates well with tracer 1, it yields significantly higher free T4 values, presumably due to the release of albumin bound T4 by the high concentration (0.1%) of sulfobromophthalein.

Oleic acid added to tracer 5 is partically capable of displacing the analog tracer from albumin. However, patient data generated with this tracer show poor correlation with data generated with tracer 1, given oleic acid at this concentration. Higher concentrations of oleic acid in the tracer-concentrations greater than 1.0 mmol/l-displace bound unlabeled T4 from albumin.

Example 6: To examine the effects of nonesterified free fatty acids on the free T4 and free T3 assay systems, patient samples were aliquoted, lyophilized, and then reconstituted with different concentrations of oleic acid in distilled water. The reconsituted samples were assayed for free T3 and free T4 according to the protocol given above, using the same four tracers described in Example 3. The results, summarized in Table 18, indicate clearly that tracer 1 for

free T4 is substantially bound to albumin, and that the addition of oleic acid displaces the tracer from albumin, producing spuriously low free T4 results. Tracers II and III are also bound to albumin, but to a must lesser degree. Tracer IV, however, is essentially unaffected by albumin, as shown in Example 3; moreover, oleic acid has no significant effect on free T4 values.

Results for free T3 are similar to those for free T4 in that they show tracer IV to be essentially unaffected by nonesterified free fatty acids, again confirming the results obtained in Example 3.

Table 18. Effect of Oleic Acid

	Free T4 Tracer				Free T3 Tracer			
	I	II	III	IV	I	II	III	IV
Neat	1.1	1.0	1.7	1.4	3.9	3.6	4.5	5.3
+ 2.5 mmol/l	0.4	0.9	1.1	1.3	2.4	3.2	3.7	5.3
+ 5.0	0.3	0.9	1.1	1.3	2.7	2.9	4.2	5.3
+ 7.5	0.3	0.8	1.1	1.3	3.2	2.8	4.2	5.5
+ 10.0	0.4	0.7	1.2	1.2	3.0	3.0	4.4	5.1
	n = 3	n = 3	n = 4	n = 4	n = 3	n = 3	n = 4	n = 4

Example 7: In order to establish that the results generated by the free T4 assay described above is unaffected by pregnancy and in non-thyroidal illness, 185 euthyroid samples were assayed using the tracer IV described in Example 3 above and compared to 25 first-trimester and 49 third-trimester pregnancy samples, and 14 samples from non-thyroidal illness patients. The results, summarized in Table 19 and Figuress 17-20, show that there are no statistical or clinically significant differences in free T4 values during pregnancy or non-thyroidal illness as compared to a euthyroid population.

This again confirms the fact that when using appropriate albumin blocking reagents the free T4 assay is unaltered by in vivo changes in albumin concentrations.

Table 19. Free T4.

	95%	Median	N
Euthyroids	0.8 - 2.0	1.3	185
Pregnancy			
1st trimester	0.9 - 2.2	1.5	25
3rd trimester	0.7 - 2.1	1.5	49
NTI	0.8 - 1.9*	1.2	14

*Absolute range

Free Testosterone

It is well established from prior art that steroid molecules bind to their natural binders through the A and/or B ring of the molecule. See Forest, M., et al and references therein (Free and bound steroids in plasma: methodology and physiopathological implications, In: Physiological Peptides and New Trends in Radioimmunology, C.A. Bizollon, ed., Amsterdam: Elsevier/North-Holland Biochemical Press, 1981, 249-266.) Chemical alteration of the A and/or B ring will inhibit most steroids - including testosterone, progesterone, estradiol, cortisol, and so on - from binding to endogenous binders. Testosterone was selected as a representative member of this family. A testosterone analog, 6-hydroxytestosterone-19-carboxymethyl ether histamine, was systhesized and radiolabeled with iodine 125 by conventional techniques. This analog tracer was subsequently compounded in 0.01M HEPES buffer, pH = 7.4, containing 1 mg/ml charcoal-absorbed human serum albumin and 0.01% sodium azide. Blocking agents were added, as described in the specific examples below.

Antibodies to testosterone were raised in rabbits using testosterone-19-carboxymethyl ether bovine serum albumin as the immunogen, and immobilized on the inner walls of polypropylene 12x75mm tubes as described above for free T4 and free T3. Free testosterone calibrators, prepared by adding different amounts of testosterone to human serum free of any apparent testosterone, were calibrated by direct equilibrium dialysis and assigned free testosterone values in pg/ml. For the assay of free testosterone, 50 µl of calibrator or patient sample is pipetted into antitestosterone antibody-coated tubes, followed by the addition of 1.0 ml of iodinated 6-hydroxy-testosterone-19-carboxymethyl ether histamine analog. The tubes are incubated for 4 hours at 37°C, then decanted and radioactivity counted. Results are computed by interpolation from the calibration curve.

Free Testosterone Examples

Example 1: To investigate the effect of blocking agents on free testosterone results, twenty samples were assayed for free testosterone using iodinated analog - compounded as described above - both with and without sulfobromophthalein (SBP), and with various amounts of sodium salicylate, 2,4-dinitrophenol (DNP) and 8-anilino-1-naphthalenesulfonic acid (ANS). Mean values for each tracer, in pg/ml, are summarized below.

Table 20.

Tracer	Buffer	- 5 Salicylate	- 10 Salicylate	+0.15 DNP	- 0.3 DNP	- 1.0 ANS	- 2.0 ANS
Without SBP	7.0 (A)	6.0 (B)	14.3 (C)	10.5 (D)	13.4 (E)	18.9 (F)	17.7 (G)
With SBP	7.1 (A)	13.1 (B)	14.4 (C)	9.8 (D)	13.5 (E)	16.8 (F)	15.0 (G)

The regression equations between corresponding tracers are given below.

Table 21.

A = 1.14 A	- 0.21	r = 1.000
B = 1.06 B	- 0.11	r = 0.997
C = 1.02 C	- 0.31	r = 0.998
D = 1.11 D	- 0.30	r = 0.996
E = 1.03 E	- 0.32	r = 0.997
F = 1.13 F	- 0.05	r = 0.996
G = 1.19 G	- 0.26	r = 0.997
A = 2.76 F	- 2.70	r = 0.966
A = 2.34 G	- 1.58	r = 0.987

From the example above we find that the absence of sulfobromophthalein will increase the apparent free testosterone levels by 14% since sulfobromophthalein inhibits the binding of the analog tracer to albumin without displacing testosterone bound to albumin. We also find - and this is of major importance - that salicylate, 2,4-dinitrophenol and ANS displace testosterone from albumin and/or SHBG, thus increasing the apparent free testosterone as measured by this method.

Example 2: In order to check the efficacy of the analog tracer in the free testosterone assay, iodinated 6-hydroxytest-osterone-19-carboxymethyl ether histamine (analog tracer) was compared to iodinated testosterone-19-carboxymethyl ether histamine (regular tracer) in assays for free testosterone in patient samples.

The tracers were compounded as described above with 10 μg/ml sulphobromophthalein. In order to maintain equivalent sensitivity, adjustments were made for each tracer in the amount of antibody immobilized onto the inner wall of the propylene tubes.

Twenty patient samples were assayed following the free testosterone protocol already described, using the two tracers mentioned above. The mean free testosterone values, in pg/ml, and the regression equation are displayed below.

Table 22.

Tracer	6-Hydroxytestosterone-19-histamine-^{125}I	Testosterone-19-histamine-^{125}I
Mean (n = 20)	11.0 (A)	17.5 (B)
	A = 1.48 B + 1.24 r = 0.977	

The results clearly indicate that the analog 6-hydroxy-testosterone-19-histamine-^{125}I tracer does not bind to endogenous binders, while the tracer tesetosterone-19-histamine-^{125}I does, thus yielding approximately 50% higher free testosterone values compared to the analog tracer under identical experimental conditions.

Example 3: To investigate the effect of sex hormone-binding globulin (SHBG) levels on the free testosterone assay system, a charcoal-absorbed human serum pool was spiked with 400 μg SHBG/milliliter, a level which is approximately 10 times normal. The SHBG-spiked pool, when assayed by the free testosterone procedure, showed a percent bound value of 99% B/B_o.

Since charcoal absorption removes testosterone from the serum pool, it should have free (and total) testosterone concentrations of zero - that is, percent bound values of approximately 100% B/B_o - both before and after spiking. The results show, as desired, that the analog tracer, 6-hydroxy-testosterone-19-histamine-^{125}I, does not bind to even high levels of SHBG.

Example 4: In order to investigate the effect of elevated albumin levels on the free testosterone procedure, three lyophilized samples were reconstituted with aqueous solutions containing 0, 1.0, 2.0 and 3.0 gm/dl of charcoal-absorbed human serum albumin. All samples were assayed in parallel using the same tracer as in Example 3, with the following results.

Table 23.

Sample	Unspiked	Spiked with Albumin gm/dl 1.0	2.0	3.0
1	4.7	4.4	4.2	3.9
2	16.7	16.4	16.7	15.8
3	37.0	37.0	34.2	34.0
Mean	19.5	19.3	18.4	17.9
Recovery	—	99%	94%	92%

The results show that there is no clinically significant effect due to even major increases in the albumin level. Note that samples spiked with 3.0 gm/dl represent a very high level of albumin, in the order of 7 gm/dl.

Example 5: Several patient samples were analyzed by the free testosterone procedure using the same tracer as in Example 3 both before and after charcoal absorption. Displayed below are the free testosterone concentrations (in pg/ml) before charcoal absorption, and the percent bound ($\%B/B_O$) values following charcoal absorption.

Table 24.

Normal Males		Normal Females		3rd Trimester	
Before	After	Before	After	Before	After
21.24	96%	1.80	103%	4.73	99%
14.33	98%	2.32	104%	5.20	96%
19.91	96%	1.73	104%	3.58	96%
21.03	98%	3.47	104%	4.44	97%
15.01	95%	1.93	105%	3.86	96%

The results show, as desired, that charcoal absorption essentially reduces the apparent free testosterone level of patient samples, as measured by the analog procedure, to zero, that is, to percent bound values of approximately 100% B/B_o. Since charcoal absorption removes testosterone along with other steroids and small molecules from serum sample, while leaving larger molecules such as albumin, SHBG and other binding proteins, this experiment helps to confirm that the analog free testosterone procedure is not influenced by levels of the transport proteins as such.

Example 6: Since non-esterified free fatty acids (NEFA) have a higher association constant to albumin than does testosterone, addition of NEFA should displace free testosterone from albumin. This was confirmed by an experiment in which various amounts of oleic acid were added to each of three patient samples. The effects on the apparent free testosterone levels are shown in Table 24.

Table 25.

Oleic Acid Added	Patient 1	Patient 2	Patient 3	Mean
0 mmol/l	5.2	3.3	10.0	6.5
2.5	7.3	4.7	11.5	7.9
5.0	11.5	11.1	21.8	14.8
7.5	21.2	16.0	32.3	23.2
10.0	30.3	17.9	47.8	32.0

Having fully described the invention, it is intended that it be limited solely by the lawful scope of the appended claims.

CLAIMS

1. A method for measuring free ligands in biological fluids in the presence of bound ligand and endogenous binding proteins, without disturbing the equilibrium between the free ligand and the protein-bound ligand, comprised of the following steps:

(a) incubating a sample of biological fluid with (i) a ligand analog tracer which, due to its chemical structure, does not bind to some of the endogenous binding proteins, (ii) a specific ligand binder and (iii) at least one specific chemical inhibitor reagent that inhibits the binding of the ligand analog tracer to other endogenous binding proteins;

(b) separating the ligand analog tracer bound to the specific binder from unbound tracer; and

(c) determining the concentration of free ligand in said biological fluid.

2. A method for measuring free ligands in biological fluids in the presence of bound ligand and endogenous binding proteins, without disturbing the equilibrium between the free ligand and the protein-bound ligand, comprised of the following steps:

(a) incubating a sample of biological fluid with (i) a ligand analog tracer which, due to its chemical structure, does not bind to some of the endogenous binding proteins, (ii) a specific ligand binder and (iii) specific chemical inhibitor reagents that alone or in combination inhibit the binding of the ligand analog tracer to other endogenous binding proteins;

(b) separating the ligand analog tracer bound to the specific binder from unbound tracer; and

(c) comparing the bound fraction in said sample to the bound fraction of a given set of known free ligand calibrators to determine the concentration of free ligand in said biological fluid.

3. The method of Claim 1 wherein the chemical inhibitor reagent is a substituted monoaryl organic compound.

4. The method of Claim 1 wherein the other endogenous binding protein includes albumin.

5. The method of Claim 1 wherein the chemical inhibitor agent is 2,4-dinitrophenol.

6. The method of Claim 1 wherein the chemical inhibitor agent is sodium salicylate.

7. The method of Claim 1 wherein the free ligand is a hormone, steroid, drug, drug metabolite, polypeptide, protein, vitamin, antigen or toxin.

8. The method of Claim 1 wherein the free ligand is a thyroid hormone.

9. The method of Claim 1 wherein the free ligand is a sex hormone.

10. The method of Claim 1 wherein the specific ligand binder is an antibody to said free ligand.

11. The method of Claim 1 wherein the specific ligand binder is immobilized on a solid substrate.

12. The method of Claim 1 wherein the specific ligand binder is carried on a polypropylene substrate.

13. The method of Claim 1 wherein the ligand analog tracer is N-^{125}I-L-triiodothyronine succinimide.

14. The method of Claim 1 wherein the ligand analog tracer is N-^{125}I-L-thyroxine succinimide.

15. The method of Claim 1 wherein the ligand analog tracer is labeled with a radioactive atom, an enzyme, fluorophor, light chromophore or chemiluminescent group.

16. The method of Claim 1 wherein the ligand analog tracer is labeled with at least one radioactive iodine atom.

17. The method of Claim 1 wherein the free ligand is testosterone.

18. The method of Calim 1 wherein the ligand analog tracer is iodinated 6-hydroxytestosterone-19-carboxymethyl ether histamine analog.

19. The method of Claim 2 wherein said known free ligand calibrators are prepared by adding different amount of ligand to ligand - free human serum, calibrated by equilibrium dialysis and assigned free ligand values.

20. The method of Claim 1 wherein said method is carried out at about 37°C.

21. The method of Claim 1 wherein said method is carried out at about pH 7.4.

22. The method of Claim 1 wherein the chemical inhibitor reagent is a dye.

23. The method of Claim 1 wherein the chemical inhibitor reagent is sulfobromophthalein.

24. The method of Claim 1 wherein the chemical inhibitor reagent is a fatty acid.

25. The method of Claim 1 wherein the chemical inhibitor reagent is oleic acid.

26. The method of Claim 1 wherein the chemical inhibitor reagent is a phenolic hydroxyl compound.

27. The method of Claim 1 wherein the chemical inhibitor reagent is an amino acid.

ABSTRACT

A method for measuring free ligands in biological fluids in the presence of bound ligand and endogenous binding proteins, without disturbing the equilibrium between the free ligand and the protein-bound ligand, comprised of the following steps: (a) incubating a sample of biological fluid with (i) a ligand analog tracer which, due to its chemical structure, does not bind to some of the endogenous binding proteins, (ii) a specific ligand binder and (iii) specific chemical inhibitor reagents that alone or in combination inhibit the binding of the ligand analog tracer to other endogenous binding proteins; (b) separating the ligand analog tracer bound to the specific binder from unbound tracer; and (c) comparing the bound fraction in said sample to the bound fraction of a given set of known free ligand calibrators to determine the concentration of free ligand in said biological fluid.

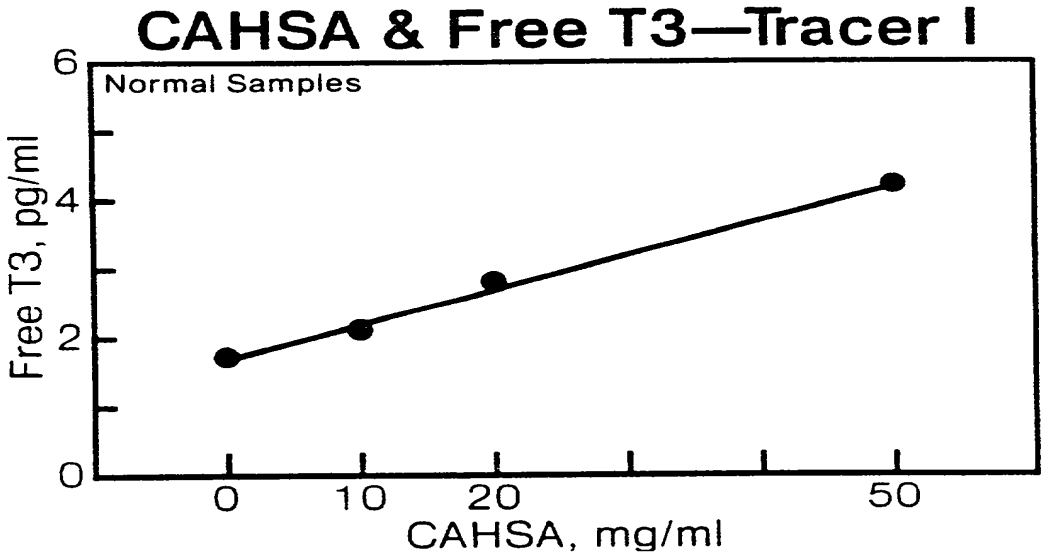
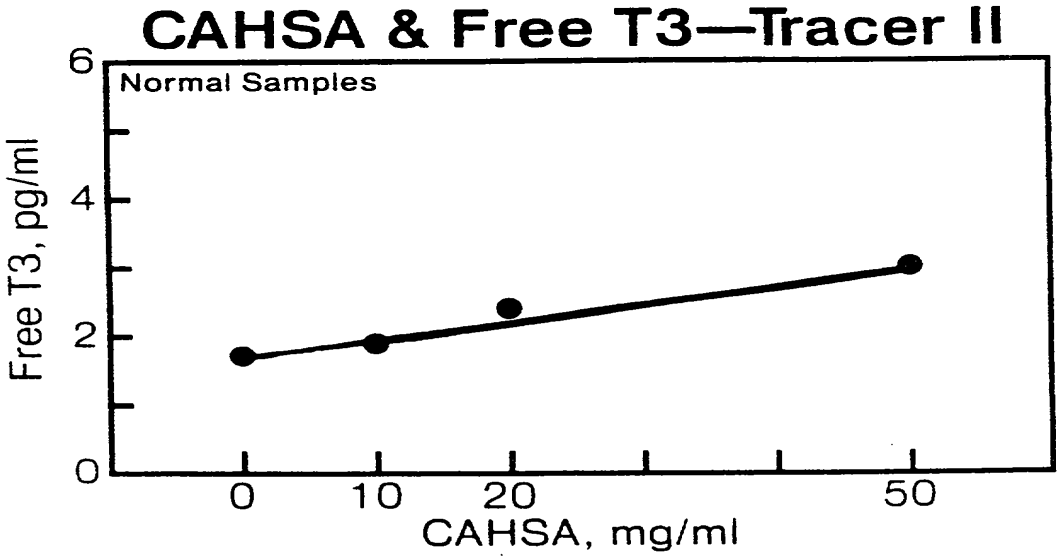

CAHSA & Free T3—Tracer I
Normal Samples
Free T3, pg/ml
6
4
2
0
0
10
20
50
CAHSA, mg/ml

FIG. 1.


CAHSA & Free T3—Tracer II
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Free T3, pg/ml
6
4
2
0
0
10
20
50
CAHSA, mg/ml

FIG. 2.

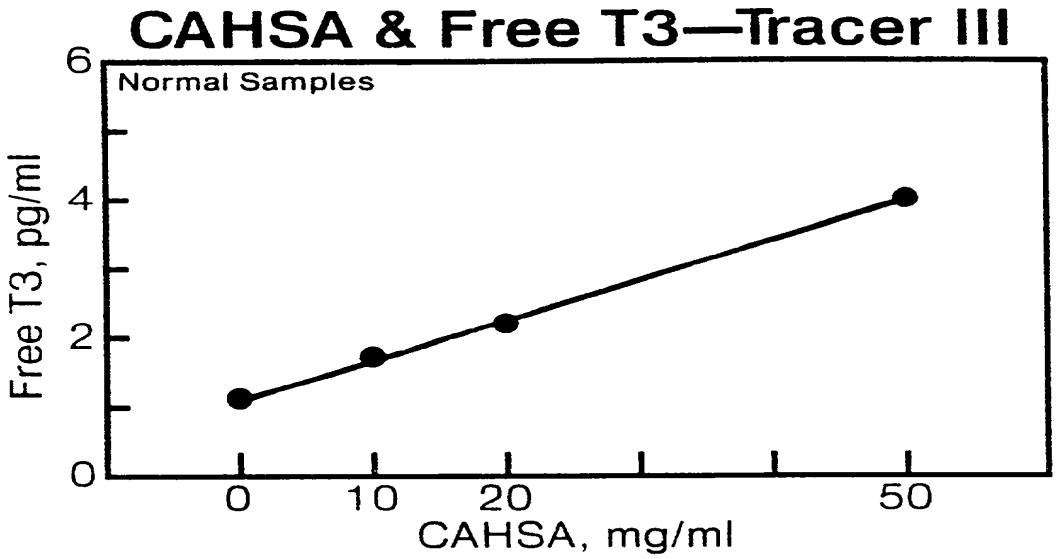
CAHSA & Free T3—Tracer III
Normal Samples
Free T3, pg/ml
6
4
2
0
0
10
20
50
CAHSA, mg/ml

FIG. 3.

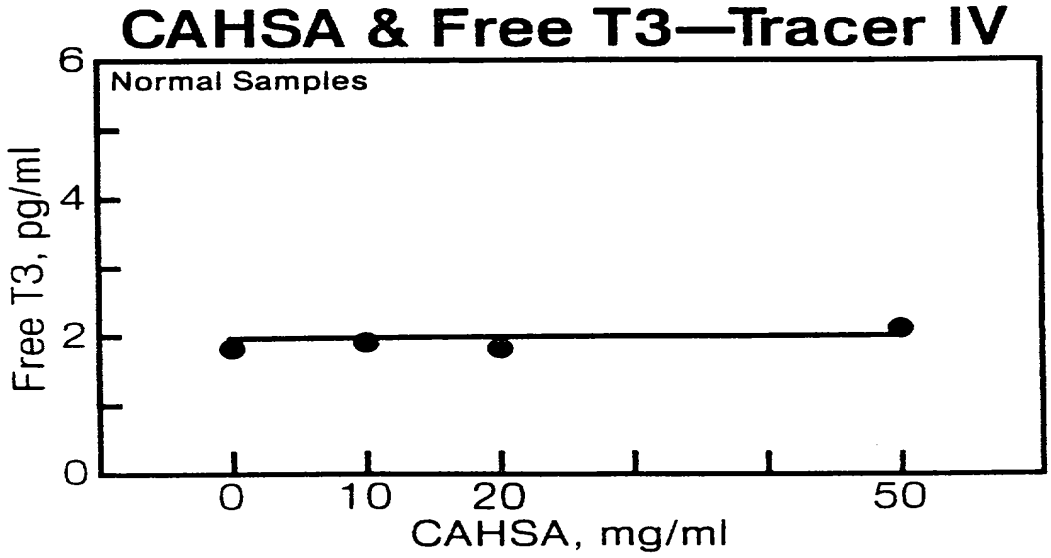
CAHSA & Free T3—Tracer IV
Normal Samples
Free T3, pg/ml
6
4
2
0
0
10
20
50
CAHSA, mg/ml

FIG. 4.

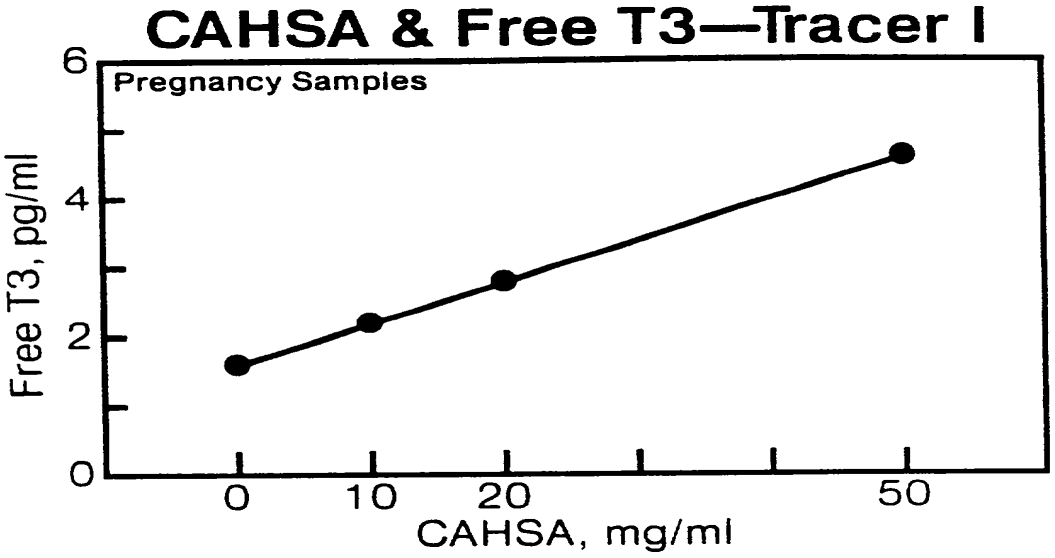
CAHSA & Free T3—Tracer I
Pregnancy Samples
Free T3, pg/ml
6
4
2
0
0
10
20
50
CAHSA, mg/ml

FIG. 5.

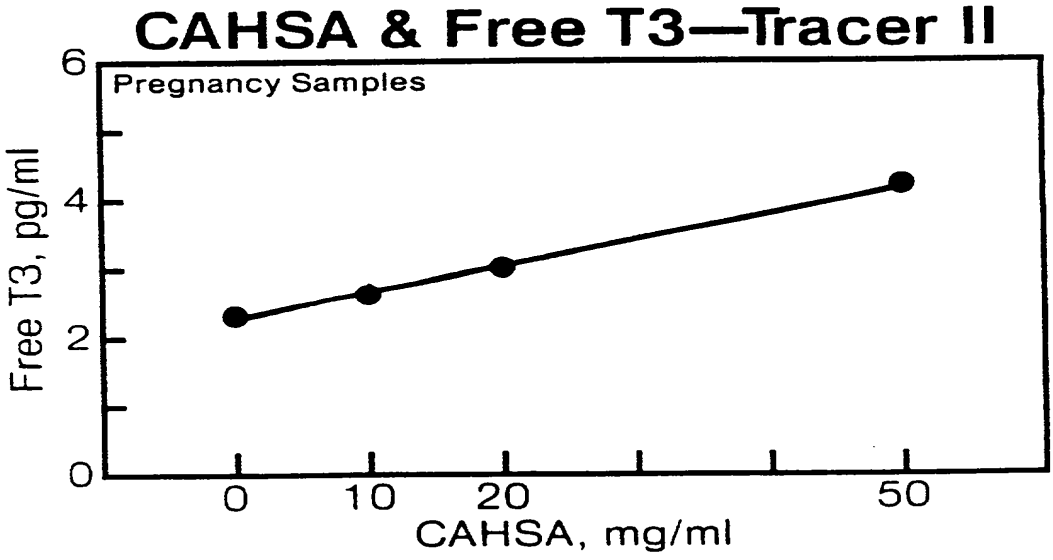
CAHSA & Free T3—Tracer II
Pregnancy Samples
Free T3, pg/ml
6
4
2
0
0
10
20
50
CAHSA, mg/ml

FIG. 6.

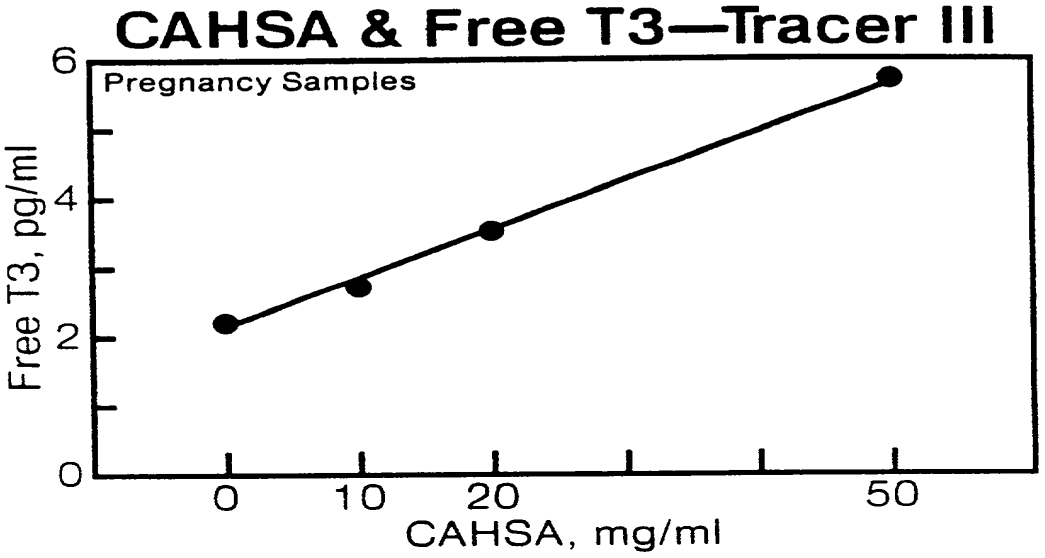
CAHSA & Free T3—Tracer III
Pregnancy Samples
Free T3, pg/ml
6
4
2
0
0
10
20
50
CAHSA, mg/ml

FIG. 7.

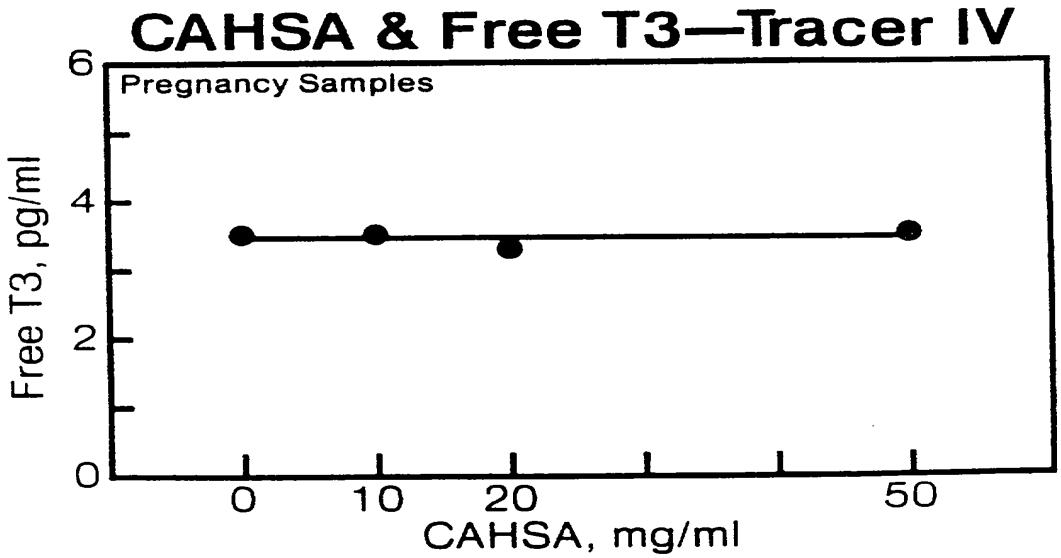
CAHSA & Free T3—Tracer IV
Pregnancy Samples
Free T3, pg/ml
6
4
2
0
0
10
20
50
CAHSA, mg/ml

FIG. 8.

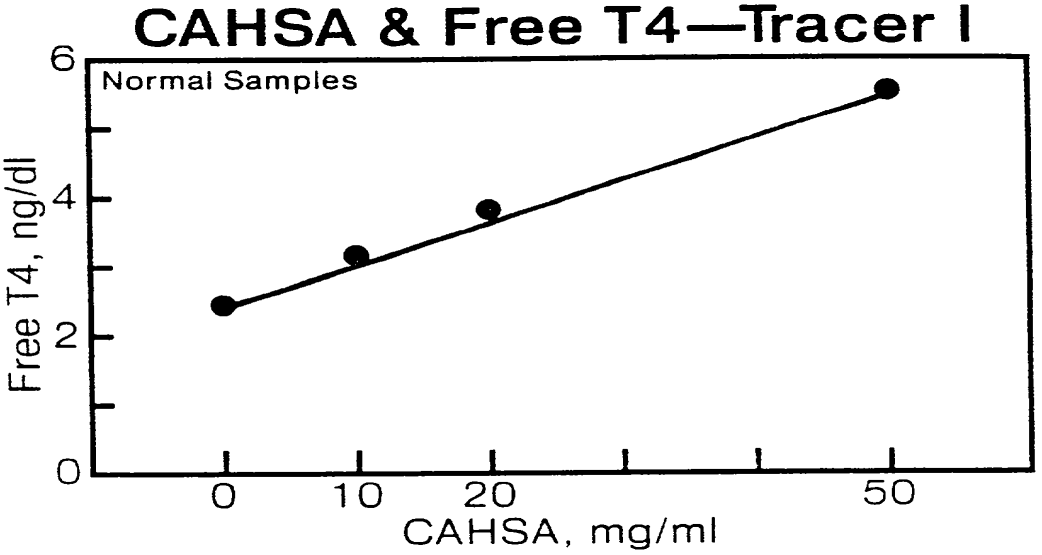
CAHSA & Free T4—Tracer I
Normal Samples
Free T4, ng/dl
6
4
2
0
0
10
20
50
CAHSA, mg/ml

FIG. 9.

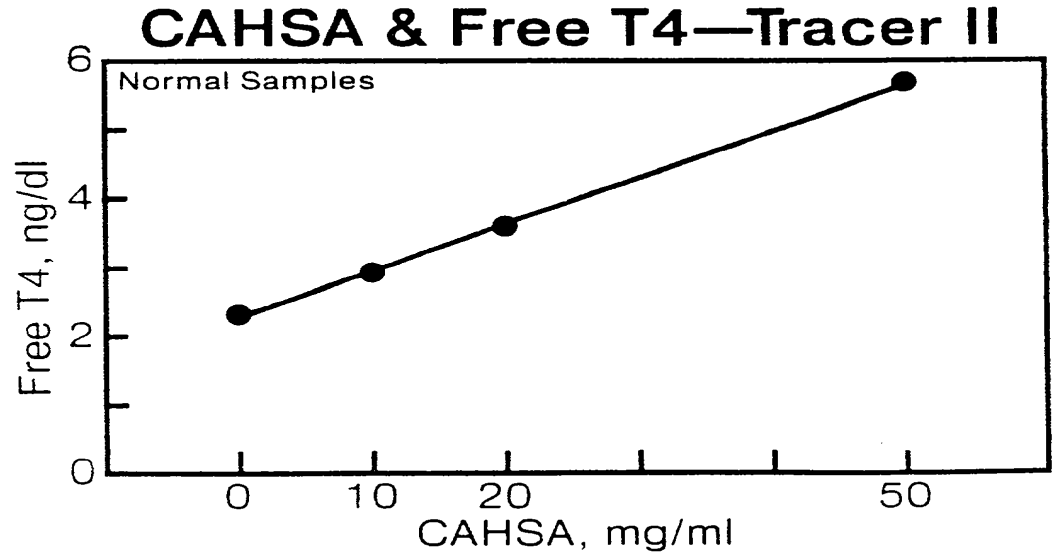
CAHSA & Free T4—Tracer II
Normal Samples
Free T4, ng/dl
6
4
2
0
0
10
20
50
CAHSA, mg/ml

FIG. 10.

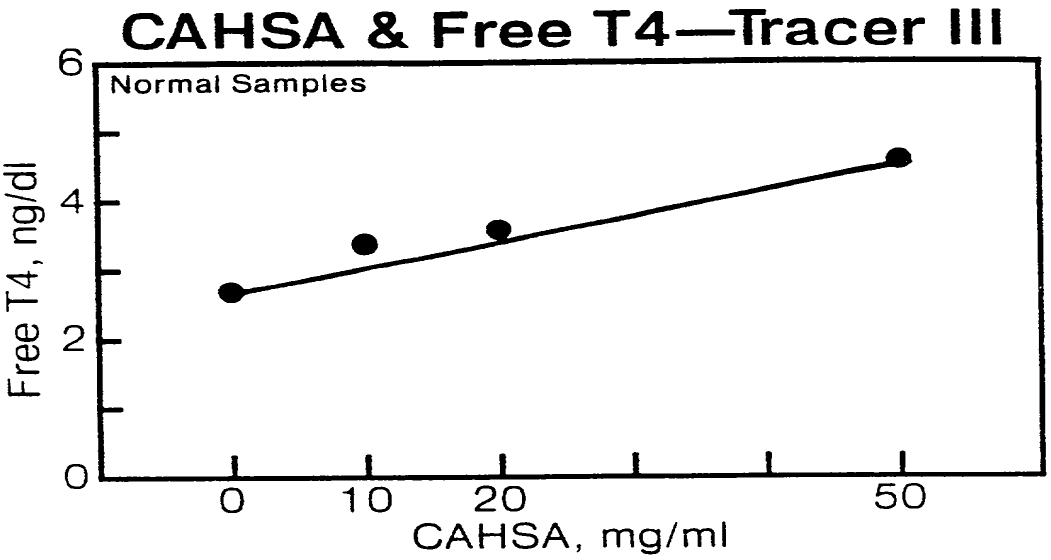
CAHSA & Free T4—Tracer III
Normal Samples
Free T4, ng/dl
6
4
2
0
0
10
20
50
CAHSA, mg/ml

FIG. 11.

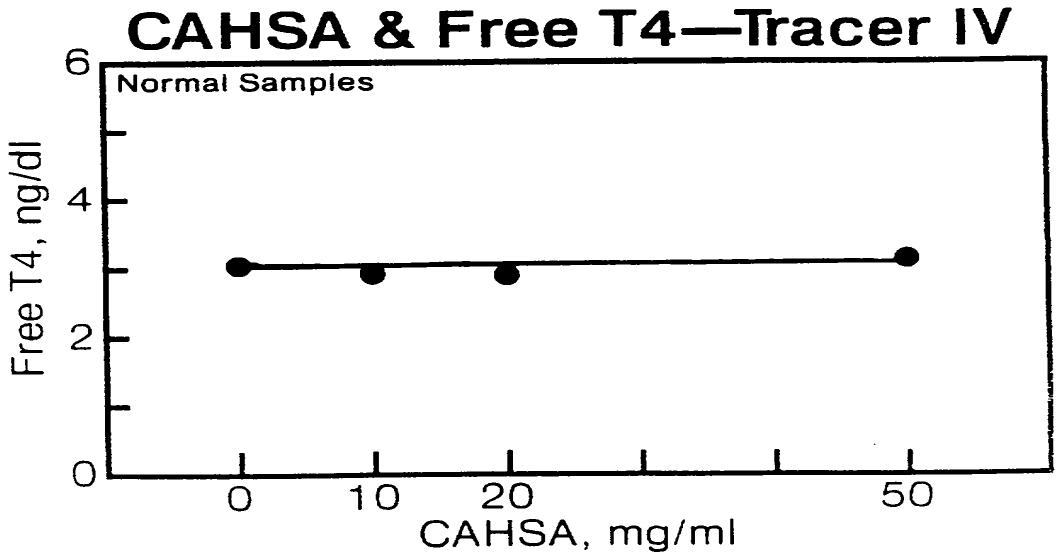
CAHSA & Free T4—Tracer IV
Normal Samples
Free T4, ng/dl
6
4
2
0
0
10
20
50
CAHSA, mg/ml

FIG. 12.

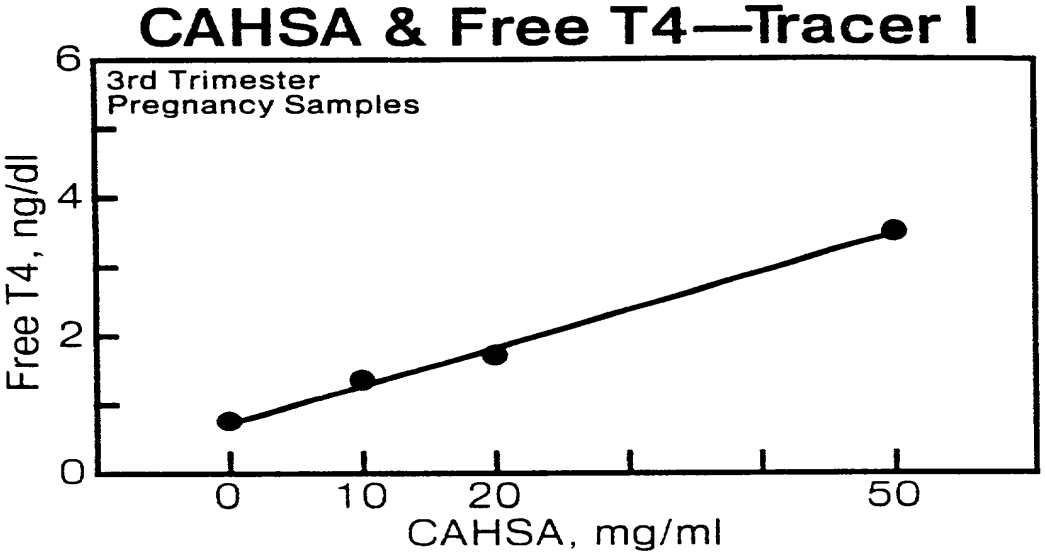
CAHSA & Free T4—Tracer I
3rd Trimester
Pregnancy Samples
Free T4, ng/dl
6
4
2
0
0
10
20
50
CAHSA, mg/ml

FIG. 13.

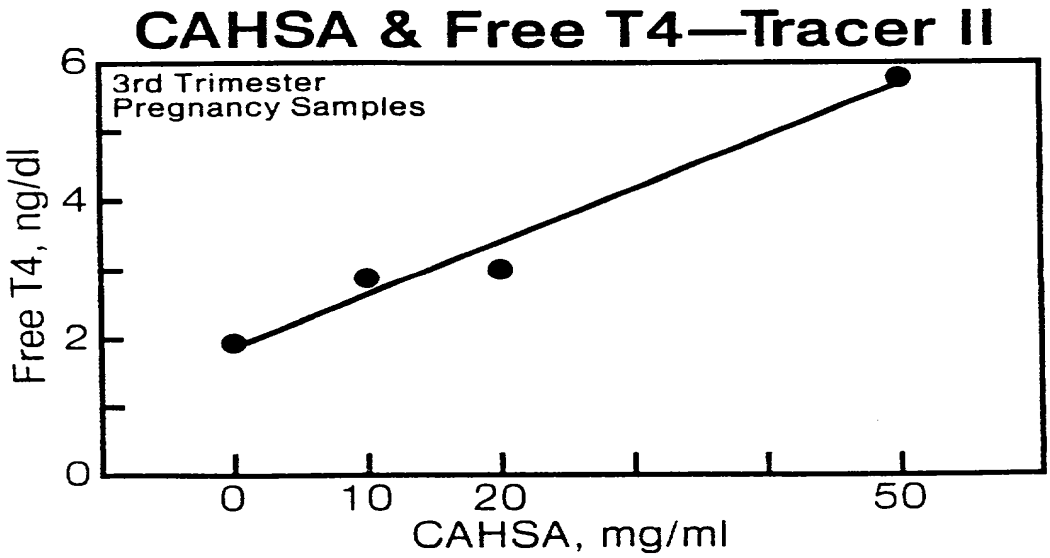
CAHSA & Free T4—Tracer II
3rd Trimester
Pregnancy Samples
Free T4, ng/dl
6
4
2
0
0
10
20
50
CAHSA, mg/ml

FIG. 14.

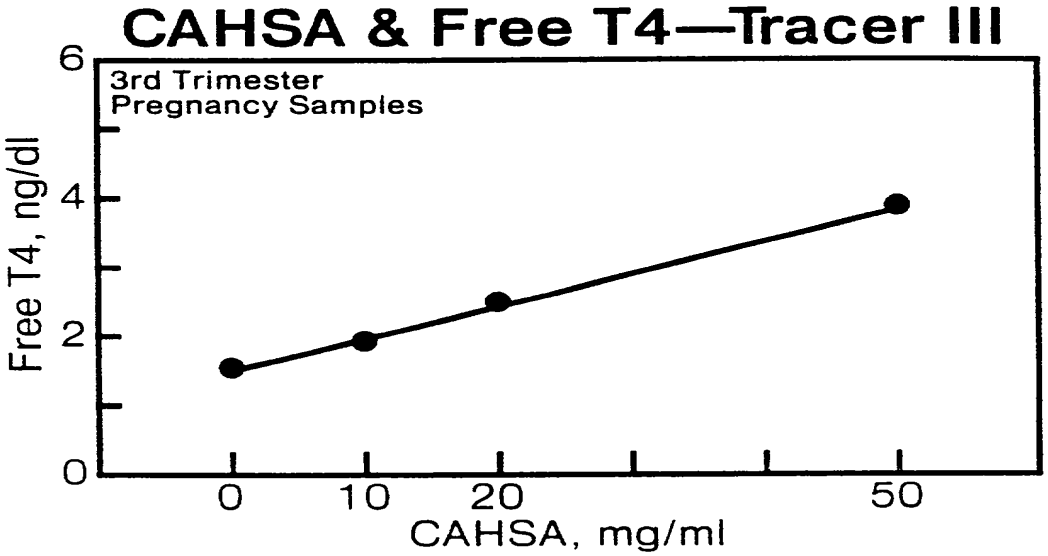
CAHSA & Free T4—Tracer III
3rd Trimester
Pregnancy Samples
Free T4, ng/dl
6
4
2
0
0
10
20
50
CAHSA, mg/ml

FIG. 15.

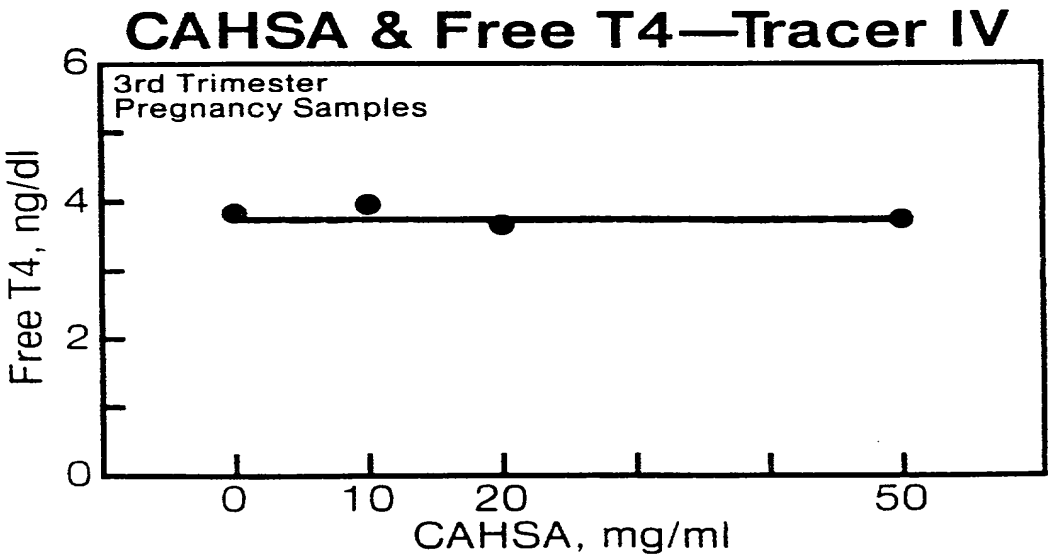
CAHSA & Free T4—Tracer IV
3rd Trimester
Pregnancy Samples
Free T4, ng/dl
6
4
2
0
0
10
20
50
CAHSA, mg/ml

FIG. 16.

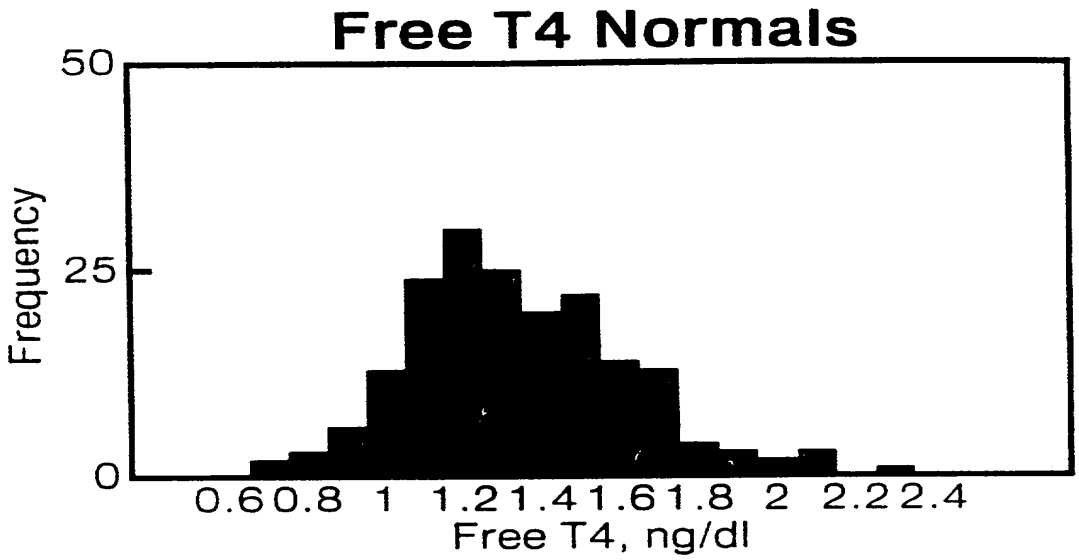
Free T4 Normals
50
25
0
Frequency
0.6 0.8 1 1.2 1.4 1.6 1.8 2 2.2 2.4
Free T4, ng/dl

FIG. 17.

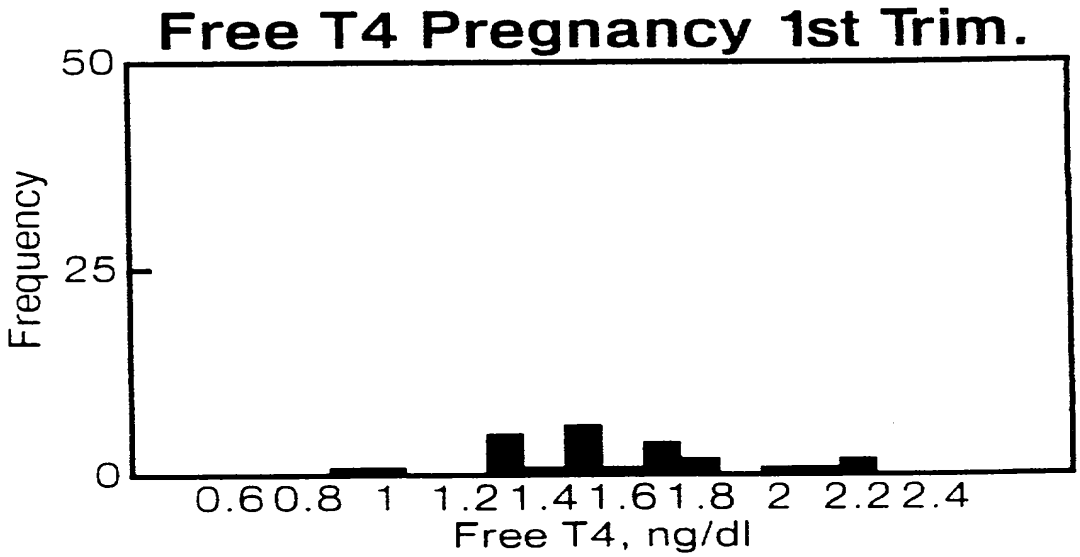
Free T4 Pregnancy 1st Trim.
50
25
0
Frequency
0.6 0.8 1 1.2 1.4 1.6 1.8 2 2.2 2.4
Free T4, ng/dl

FIG. 18.

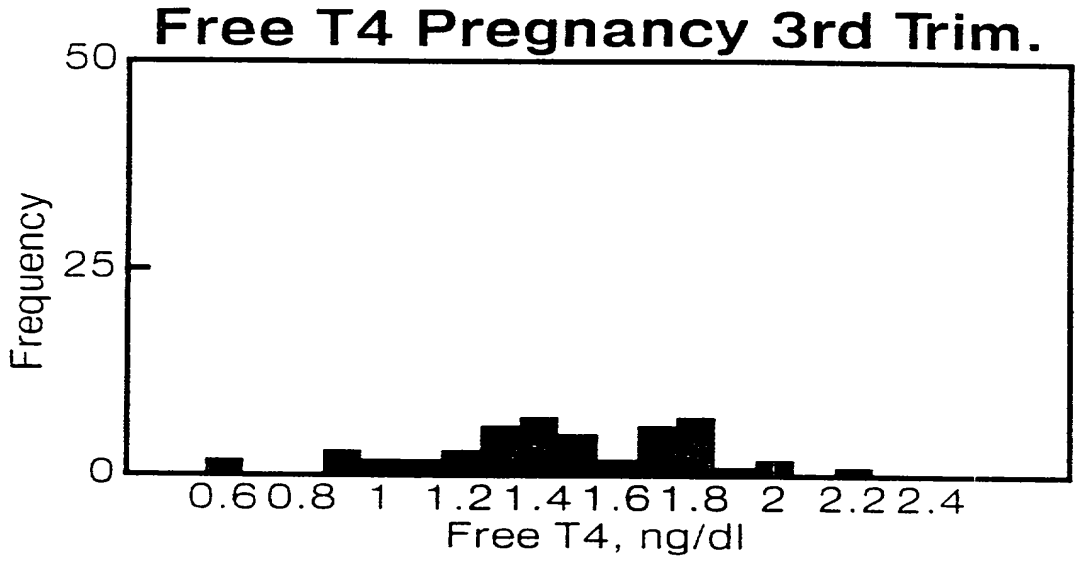
Free T4 Pregnancy 3rd Trim.
50
25
0
Frequency
0.6 0.8 1 1.2 1.4 1.6 1.8 2 2.2 2.4
Free T4, ng/dl

FIG. 19.

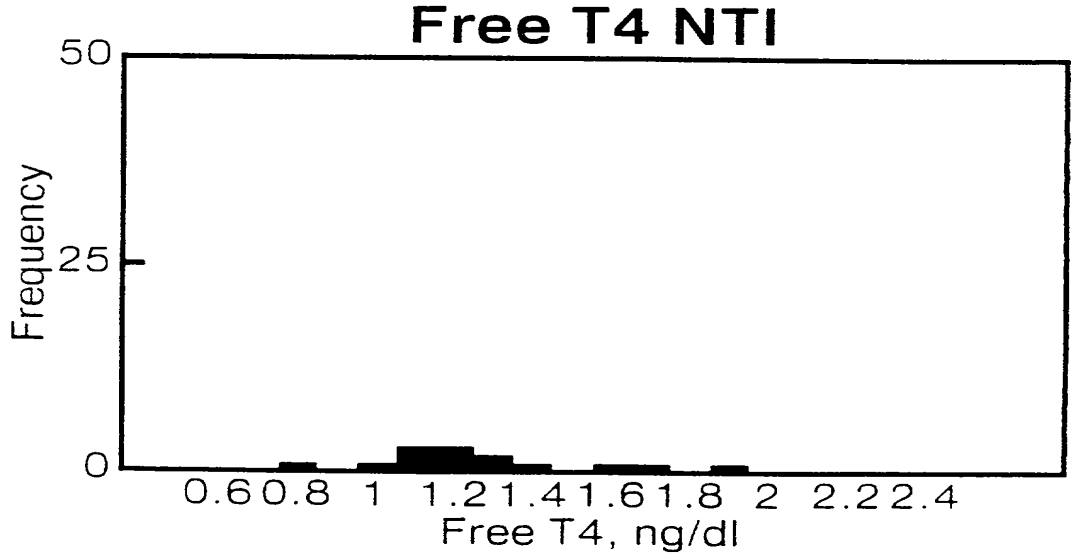
Free T4 NTI
50
25
0
Frequency
0.6 0.8 1 1.2 1.4 1.6 1.8 2 2.2 2.4
Free T4, ng/dl

FIG. 20.

107-145D-C

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Group Art Unit: Not Assigned
A. Said El Shami	Examining Attorney: Not Assigned
Continuation Serial No.: Not Assigned (Divisional Serial No.: 07/303,712)	
Continuation Application Filed: Herewith (Divisional Filed 01/27/1989)	Date: March 9, 1998
For: METHOD FOR MEASURING FREE LIGANDS IN BIOLOGICAL FLUIDS, AND ASSAY KITS FOR MEASURING SAME	Pasadena, California

I hereby certify that this correspondence is being deposited with the United States Postal Service as express mail in an envelope addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231 on 3/9/98

By Laura Delarde

Date 3/9/98

EXPRESS MAIL LABEL # EI262826088US

Hon. Commissioner of
Patent and Trademarks
Washington, D.C. 20231

DECLARATION OF SAID EL SHAMI

I, Said El Shami, declare:

I am the applicant in this patent application, assigned to Diagnostic Products Corporation ("DPC").

The Board raised the question of public use more than one year prior to October 4, 1985, the filing date of the ultimate parent application, United States Patent Application Serial Number 784,857. DPC first began the use of blocking agents in analog based assays for free hormones in 1982. The first product, which

was released on July 15, 1982, contained 0.5% salicylate at 1X concentration as a selective blocker for albumin in a Free T4 assay. Free T3 assay was released on February 4, 1983 and also contained sodium salicylate as the selective blocker for albumin. However, these products and all other products released prior to October 4, 1984 did not contain an antibody (ligand binder) that was of an affinity and at a concentration effective to avoid stripping of T_3 and T_4 off of endogenous proteins. Consequently, these products did not truly measure free hormone levels and did not come within the scope of the instant claims.

The general theory behind the use of blockers was orally presented at the Tenovous Workshop on Quality Control held in Cardiff, Wales, on September 4, 1984. This work was then released to the public on October 4, 1984 through a publication by DPC (ZE001-320A) and later was published in the Communications in Laboratory Medicine Volume 1, No. 3, page 97 1985 (July 1985).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the

application, any patent issuing thereon, or any patent to which this verified statement is directed.

Said El Shami

DECLARATION FOR PATENT APPLICATION

Docket No. 107-145

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled METHOD FOR MEASURING FREE LIGANDS IN BIOLOGICAL FLUIDS, AND ASSAY KITS FOR MEASURING SAME, the specification of which

(check one) X is attached hereto.
___ was filed on _____ as Application Serial No. _____ and was amended on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I do not know and do not believe that the same was ever known or used in the United States of America before my invention thereof, or patented or described in any printed publication in any country before my invention thereof or more than one year prior to this application, that the same was not in public use or on sale in the United States of America more than one year prior to this application, and that the invention has not been patented or made the subject of an inventor's certificate issued before the date of this application in any country foreign to the United States of America on an application filed by me or my legal representatives or assigns more than twelve months prior to this application.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			Priority Claimed	
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) listed below and, insofar as the subject matter of the claim of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Section 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing Date)	(Status-patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status-patented, pending, abandoned)

I hereby appoint JOSEPH E. MUETH, Registration No. 20,532 with offices located at 700 South Flower Street, Suite 2200, Los Angeles, California 90017, telephone (213) 688-7407, my attorney with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor A. Said El Shami

Inventor's signature A. Said El Shami Date Oct. 4, 1985

Residence Agoura Hills, Calif. Citizenship U.S.A.

Post Office Address 29974 Rolling Ridge Drive
Agoura Hills, California 91301

Full name of second joint inventor, if any _____

Second Inventor's signature _____ Date _____

Residence _____ Citizenship _____

Post Office Address _____

Full name of third joint inventor, if any _____

Third Inventor's signature _____ Date _____

Residence _____ Citizenship _____

Post Office Address _____